EUK-b106117- 6-P25829

# British Journal of Pharmacology

## INDEX

(1966–1973)

The eight year cumulative index (1966–1973) to the British Journal of Pharmacology is now available.

Price £10.45 (including postage)

Available from:

DEPARTMENT AS11,
MACMILLAN JOURNALS LTD.,
BRUNEL ROAD,
BASINGSTOKE, RG21 2XS
HAMPSHIRE, ENGLAND.

Orders must be accompanied by
made payable to Macmillan Jour
in any currency at the current

emittance. Cheques should be
nals Ltd. Payment may be made
exchange rate.

# DRUGS AND CENTRAL SYNAPTIC TRANSMISSION

**Edited by P. B. Bradley and B. N. Dhawan**

*acmillan Pharmacology Monographs*

Even though drugs have been used as research tools in pharmacological, physiological and neurochemical studies for a long time, the symposium reported in this book was perhaps the first interdisciplinary meeting where this particular use of drugs was the main theme. Disciplines represented include electrophysiology, neurochemistry, biochemistry and behavioural sciences.

about £12.50 May 1976

For further details please write to Anne Calcott, The Macmillan Press, Little Essex Street, London WC2R 3LF.

M MACMILLAN PRESS

British

British Jo

# Journal of Pharmacology

ırnal of Pharmacology provides up-to-date research on all aspects
ction essential to those involved in pharmacology. Ensure you
s information each month by taking a subscription.

ın Dept, Macmillan Journals Ltd, Brunel Road, Basingstoke, Hants
. England.

ter me for a year's subscription to the BRITISH JOURNAL OF
:OLOGY starting with the issue dated . . . . . . . . . . . .

ayment of £45.00 (USA & Canada $99. Rest of world $92).

at time of publication. Payment may be made in any currency at the current exchange
can only be processed if accompanied by the correct remittance. Cheques should be
to Macmillan Journals Ltd.

. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Registered No: 785998 England Registered Office: 4 Little Essex St., London WC2R 3LF

# FITC-DEXTRANS

## for permeabili ty and microcirculatio n studies in animals er substances

### Well characterized trac volumes

- fluorescein label for easy detection
- ultra-micro determination in nanolitre sample
- narrow molecular weight distribution
- five fractions between 3,000 and 150,000 daltons

FITC-Dextrans and other products for biological research a e described in the booklet "Biomedical Research. Product guide", free on request.

Pharmacia (Great Britain) Ltd.
Paramount House,
75 Uxbridge Road,
London W5 5SS

Pharmacia
Fine Chemicals

## Textbook of Adverse Drug Reactions

Edited by D. M. Davies

The growing importance of adverse drug reactions has created the need for this comprehensive textbook. It deals with each system of the body in turn, and covers the history, epidemiology, and basic pharmacology of adverse drug reactions; their detection, investigation and treatment; damage to human chromosomes and to the foetus and infant, and all other aspects of this important subject.
£10 *Oxford Medical Publications forthcoming*

## A Handbook of Drug and Alcohol Abuse

The Biomedical Aspects

Frederick G. Hofmann and Adele D. Hofmann

This book meets the need for a comprehensive source of pharmacological and medical information on the patterns of drug abuse and their possible consequences. It gives a full biomedical description of all the drugs that are commonly abused, and discusses the possible experiences that may follow the use of each.
Paper covers £4.50 *Oxford Medical Publications*

**Oxford University Press**

## Pharmacol

Honours Graduates/F
Fellows in Pharmacol
Medicine, required fo
pharmacological resea
toxicological studies o
of potential therapeuti
Excellent opportuniti
advancement in mod
well-equipped labora
and Assurance Schem

*Application forms from the Secretary*

**Biorex Laboratories Ltd.**
Biorex House,
Canonbury Villas, Londo

CUK-H06117-6-P25829

VOLUME 58(1) SEPTEMBER 1976

# British Journal of Pharmacology

3 BUDNY, J., DOW, R.C., ECCLESTON, D., HILL, A.G. & RITCHIE, ISOBEL M. A comparison of D and L-tryptophan on the cerebral metabolism of 5-hydroxytryptamine and dopamine in the dog

9 COLLIER, H.O.J., HAMMOND, M.D. & SCHNEIDER, C. Effects of drugs affecting endogenous amines or cyclic nucleotides on ethanol withdrawal head twitches in mice

17 LUNDELL, L., SVENSSON, S.E. & NILSSON, G. Further studies on the mode of action of isoprenaline on gastric secretion in the conscious rat

27 TURNBULL, M.J. & WATKINS, J.W. Determination of halothane-induced sleeping time in the rat: effect of prior administration of centrally active drugs

37 DAVE, K.C. & DHATTIWALA, A.S. Adrenoceptors of the guinea-pig urinary bladder

43 HÖGLUND, ELIN, WESTLING, H. & WHITE, T. Circulatory effects of oral and subcutaneous administration in normal subjects of a new bronchodilator ibuterol, a pro-drug to terbutaline

47 GILLESPIE, J.S. & TILMISANY, A.K. The action of tetraethylammonium chloride on the response of the rat anococcygeus muscle to motor and inhibitory nerve stimulation and to some drugs

57 ROBERTS, FIONA & STEPHENSON, R.P. The kinetics of competitive antagonists on guinea-pig ileum

71 LULICH, K.M., MITCHELL, H.W. & SPARROW, M.P. The cat lung strip as an *in vitro* preparation of peripheral airways: a comparison of $\beta$-adrenoceptor agonists, autacoids and anaphylactic challenge on the lung strip and trachea

81 BEAL, A.M. The effect of high rates of vasopressin administration on renal potassium and sodium excretion during potassium loading in the sheep

89 BROADLEY, K.J. The release of a coronary vasodilator metabolite from the guinea-pig isolated perfused heart stimulated by catecholamines, histamine and electrical pacing and by exposure to anoxia

101 JHAMANDAS, K. & SUTAK, M. Morphine–naloxone interaction in the central cholinergic system: the influence of subcortical lesioning and electrical stimulation

109 EVANS, R.H. & SMITH, J.W. The effect of catecholamines on the influx of calcium and the development of tension in denervated mouse diaphragm muscle

117 TAYLOR, M. Effects of L-tryptophan and L-methionine on activity in the rat

121 TODA, N. Influence of dopamine and noradrenaline on isolated cerebral arteries of the dog

127 RICHARDSON, P.D.I. The actions of natural secretin on the small intestinal vasculature of the anaesthetized cat

137 BOREHAM, P.F.L. & WRIGHT, I.G. Hypotension in rabbits infected with *Trypanosoma brucei*

141 CREESE, R. & MACLAGAN, JENNIFER. Labelled decamethonium in cat muscle

149 BUNCE, K.T., PARSONS, M.E. & ROLLINGS, NITA A. The effect of metiamide on acid secretion stimulated by gastrin, acetylcholine and dibutyryl cyclic adenosine 3′,5′-monophosphate in t isolated whole stomach of the rat

**SHORT COMMUNICATION**

157 BROWN, D.A. & GARTHWAITE, J HAYASHI, E. & YAMADA, S. Action o surugatoxin on nicotinic receptors in the superio cervical ganglion of the rat

*Br. J. Pharmac.* (1976), **58**, 3–7

# A COMPARISON OF D AND L-TRYPTOPHAN ON THE CEREBRAL METABOLISM OF 5-HYDROXYTRYPTAMINE AND DOPAMINE IN THE DOG

J. BUDNY

The Proctor and Gamble Co., Miami Valley Laboratories, P.O. Box 39175,
Cincinatti, Ohio 45239, U.S.A.

R.C. DOW, D. ECCLESTON, A.G. HILL & ISOBEL M. RITCHIE

MRC Brain Metabolism Unit, University Department of Pharmacology,
1 George Square, Edinburgh EH8 9JZ

**1** After D and L-tryptophan (50 mg/kg) were given intravenously in the dog, the concentration of the amino acid was increased in ventricular cerebrospinal fluid (CSF) during the subsequent 4 h of sampling, although the concentrations were significantly lower following the administration of the D-isomer.

**2** There was no evidence that D-tryptophan increased the synthesis of 5-hydroxytryptamine (5-HT) in dog brain as judged by the failure to cause a change in 5-hydroxyindoleacetic acid (5-HIAA) concentrations in ventricular CSF different from that seen with controls.

**3** There was no appreciable conversion of D-tryptophan to L-tryptophan in the dog.

**4** D-tryptophan was cleared more rapidly from plasma than L-tryptophan.

**5** No difference in plasma binding between D and L-tryptophan was detected.

## Introduction

In exploring the effects of D-tryptophan on brain indoles in rats, Yuwiler (1973) observed that there was an increase in the cerebral 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations. Since he also observed that D-tryptophan only sparingly crossed the blood brain barrier, he attributed the rise in 5-hydroxyindole concentrations to extra-cerebral conversion of D-tryptophan to L-tryptophan via indole pyruvate. Numerous studies (du Vigneaud, Sealock & Van Etten, 1932; Berg, 1934; Oesterling & Rose, 1952) have shown that the rat is able to use D-tryptophan almost as well as L-tryptophan for growth, which is consistent with Yuwiler's explanation of increased formation of 5-hydroxyindoles from D-tryptophan. There is a likelihood of human exposure to D-tryptophan because it is sweet (Berg, 1942; Berg, 1953). It could then be considered as a substitute for the currently used artificial sweetener Saccharin, cyclamate already having been withdrawn for toxicological reasons. It is also a constituent of oral and intravenous amino acid supplements. If one then wishes to extrapolate animal studies on D-tryptophan to man, it seems likely that the rat is an inappropriate model since man is probably unable to use D-tryptophan (Baldwin & Berg, 1949; Rose, 1949). The dog on the other hand appears to be unable to convert D-tryptophan to kynurenic acid (Correll, Berg & Cowan, 1938). Furthermore, the urinary metabolite profile from dogs given D-tryptophan (L. M. Henderson and K.C. Triebwasser, personal communication) seems to be reasonably similar to the urinary metabolites of D-tryptophan from man (Hankes, Brown, Leklem, Schmaeler & Jesseph, 1972). Consequently the dog is probably a better model than the rat for examining the effect of D-tryptophan on the 5-HT pathway in animals if it is intended to extrapolate the findings to man. Therefore, we initiated these studies to determine what effect, if any, D-tryptophan had on the 5-HT pathway in the brains of dogs, and indirectly on that of dopamine.

## Methods

Four male Beagle dogs with indwelling ventricular cannulae (Ashcroft, Dow & Moir, 1968) had 0.5 ml of CSF withdrawn on 3 occasions, at 0, 30 and 60 min before an intravenous loading dose of equal volumes of 0.9% w/v NaCl solution (saline), or D or L-tryptophan (50 mg/kg made up in saline at a concentration of 20 mg/ml). This dose was chosen as that producing a change in 5-HT synthesis when cisternal CSF was used as an index of cerebral turnover (Eccleston, Ashcroft, Moir, Parker-Rhodes, Lutz & O'Mahoney, 1968). Further samples of CSF were withdrawn at 30 min, 1, 2, 3 and 4 h after the intravenous injection. Five ml of blood was removed by venepuncture at the same time as the CSF samples. The experiments were always performed at the same time of day to obviate possible errors due to circadian variation.

### *Biochemical estimations*

*Free and total tryptophan.* The 5 ml of blood was centrifuged immediately at room temperature for 5 min at 1000 *g*. The plasma was separated and an aliquot deep frozen until the total tryptophan was estimated; 1 ml of plasma was centrifuged at 1000 *g* for 1 min at room temperature through an Amicon membrane (Centriflo 224-CF-50). The ultrafiltrate was deep frozen until required for estimation (not more than 2 days). Tryptophan was then measured on 0.1 ml of plasma ultrafiltrate and 0.1 ml of CSF by a micro method based on that of Guroff & Udenfriend (1962) from the original method of Hess & Udenfriend (1959).

*Homovanillic acid (HVA) and 5-hydroxyindoleacetic acid in CSF.* These were estimated according to the spectrophotofluorescence method of Yates & Guldberg (1968) modified for small CSF volumes by Pullar, I.A. (unpublished).

### *Statistical treatment of the data*

Because the variation in the data between animals and between experiments was large, it was decided to use all the CSF data in the analysis rather than that obtained at particular time points and to treat each set as a regression problem (Kendall & Stuart, 1967). The best fitting polynomial was ascertained by computer programme and this gave information as to whether metabolites rose significantly, if they rose in a linear fashion or if they rose and fell. Such treatment of data in similar previous experiments in cisternal fluid had indicated a rise and fall of 5-HIAA after treatment with L-tryptophan (Eccleston *et al.*, 1968).

## Results

### *Tryptophan*

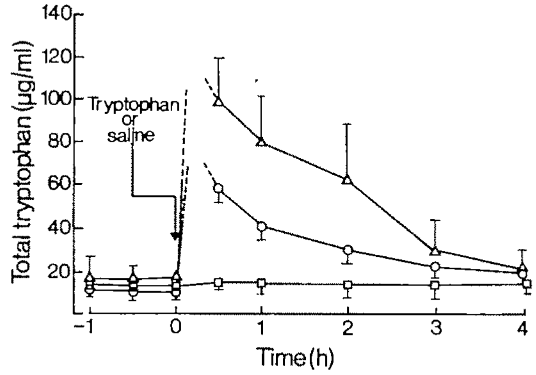


**Figure 1** Total concentration of tryptophan (free and bound) in plasma of dog both before and after an intravenous dose of D (○) or L-tryptophan (△) (50 mg/kg) or saline (□). Values are mean of 4 experiments; vertical lines show s.d.

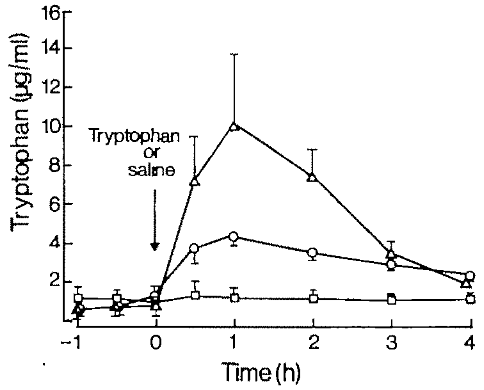


**Figure 2** Concentration of tryptophan in ventricular CSF of dog before and after an intravenous injection of D (○) or L-tryptophan (△) (50 mg/kg) or saline (□). Values are mean of 4 experiments; vertical lines show s.d.

The administration of D and L-tryptophan intravenously led to an elevation of the amino acid both in plasma and CSF (Figures 1 and 2). Even though equal quantities of the isomers were given, after 30 min and 1 h the total concentration of D-tryptophan in plasma was significantly ($P<0.02$ and $P<0.025$ respectively, Student's *t* test) lower than that for L-tryptophan and at 1 h the concentration of free tryptophan was lower for the D-isomer than the L-isomer ($P<0.05$, Student's *t* test). There was no difference in the percentage of amino acid bound to the plasma protein between the D and L forms. Taking the best fit cubic equations for the results with CSF (significance of fit $P<0.001$) the concentration of D-tryptophan peaks at 1.3 h with a value of 4.3 ± 0.3 µg/ml and the concentration of the L-isomer peaks (significance of fit cubic equation $P<0.01$) at a similar time, 1.2 h reaching 9.9 ± 1.1 µg/ml which is

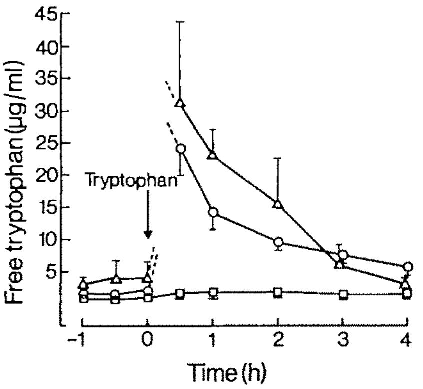


**Figure 3** Concentration of free tryptophan in plasma of dog after an intravenous dose of D (O) or L-tryptophan (△) (50 mg/kg) or saline (□). Values are mean of 4 experiments; vertical lines show s.d.

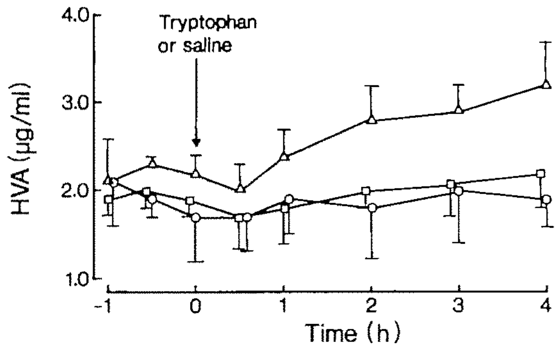


**Figure 5** Concentration of homovanillic acid (HVA) in ventricular CSF of dog after an intravenous injection of D (O) or L-tryptophan (△) (50 mg/kg) or saline (□). Values are mean of 4 experiments; vertical lines show s.d.

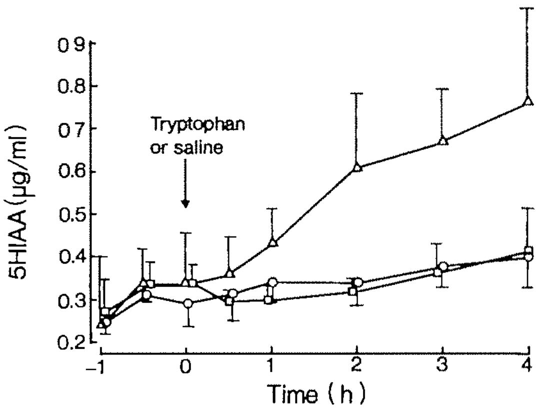


**Figure 4** Concentration of 5-hydroxyindoleacetic acid (5-HIAA) in ventricular CSF of dog after an intravenous injection of D (O) or L-tryptophan (△) (50 mg/kg) or saline (□). Values are mean of 4 experiments; vertical lines show s.d.

significantly different from the maximum concentration of D-tryptophan at the 0.001 level (Student's $t$ test). This suggests that there is no delay in entry of D-tryptophan into CSF compared with L and that the lower concentrations are a reflection of the lower concentrations of the amino acid in plasma. In the experiments in which saline was injected there was neither a change in free or total tryptophan in blood or plasma with time (Figures 1 and 3) nor in CSF (Figure 2).

### *5-Hydroxyindoleacetic acid and homovanillic acid*

The data for 5-HIAA in CSF (Figure 4) after both L-tryptophan and saline significantly fits a linear equation ($P < 0.005$ and $< 0.02$ respectively) whilst for D-tryptophan there is a linear trend which did not reach statistical significance ($0.1 > P > 0.05$). Inspection of the data (Figure 4) shows the results with D-tryptophan and saline to be initially superimposible and certainly not significantly different from each other. The rate of rise was, however, higher for L-tryptophan than saline ($P < 0.001$). In the case of HVA in CSF (Figure 5), only L-tryptophan produced a linear rise ($P < 0.001$), saline just failing to reach significance ($0.1 > P > 0.05$).

## Discussion

The aim of the present experiments was to determine whether or not there is an increase in 5-HT synthesis in the dog after the administration of D-tryptophan as has been shown in the rat (Yuwiler, 1973). It was hoped to use the technique of tryptophan loading with cisternal CSF sampling as used in previous experiments (Eccleston *et al.*, 1968). Unfortunately in preliminary experiments the data showed wide variation and ventricular CSF studies were initiated. It was found that after L-tryptophan there was a sustained rise in 5-HIAA concentration in ventricular CSF (Figure 4) and this continued throughout the 4 h of the experiment, the data fitting linear rather than quadratic equations. The previous experiments examined the concentration of 5-HIAA in both brain and cisternal CSF and showed peaks in both concentrations at about 2.3 h following the administration of L-tryptophan with a return to normal at 4 hours. The difference between the CSF from the two sites, ventricle and cisterna, may lie in the proximity of the cisterna to the choroid plexus of the 4th ventricle, the site at which acid metabolites are actively removed from the CSF (Ashcroft *et al.*, 1968). The results suggest that ventricular CSF levels of metabolites remain high after brain concentrations have fallen.

In the rat Yuwiler (1973) found that D-tryptophan penetrated brain only slowly compared with L-tryptophan and although concentrations of tryptophan in brain were almost as high after peripheral injection of the D-isomer as after the L-isomer, an analysis of the amino acid present in the brain showed it to be almost entirely L, suggesting a rapid peripheral conversion of D-tryptophan to L-tryptophan with subsequent uptake into brain. Under these circumstances a rise in cerebral 5-HT synthesis was anticipated and, in the case of the rat, found.

Our results in the dog are clearly different from the rat since there is no rise in the synthesis of 5-HT after D-tryptophan administration. After injection of the D-isomer, the concentration of tryptophan is subsequently increased in the ventricles but not after injection of saline. After both these treatments there was no rise in 5-HIAA. When L-tryptophan was given there was a rise in the concentration of both tryptophan and 5-HIAA in the ventricular CSF. This suggests that the tryptophan isomer found in the CSF after infusion of tryptophan is the D-form and supports the view that the peripheral conversion of D-tryptophan to L-tryptophan is small. A further point in the failure of D-tryptophan to raise 5-HT synthesis in these experiments is the stereo-specific nature of the hydroxylase enzyme being greatly in favour of the L-form (Gal, Armstrong & Ginsberg, 1966).

Clearance from the periphery of the two isomers was also different. The plasma concentration of D-tryptophan, both total and free at the earliest time of sampling, 30 min after administration, is lower than the plasma concentration of tryptophan measured after administration of the L-isomer and is presumably due to the more rapid renal clearance of D-tryptophan, perhaps because of failure to reabsorb the D-isomer from the glomerular filtrate. Differences in the binding of D-tryptophan compared with L-tryptophan to plasma proteins were not apparent in this study. This is in conflict with the findings of McMenamy & Oncly (1958) who demonstrated that D-tryptophan had essentially no affinity for human serum albumin. However, these experiments were *in vitro* measurements done at equilibrium (5 h and 2°C) experimental conditions which differed substantially from our own.

After L-tryptophan there was also a massive rise in the concentration of HVA in ventricular CSF. This phenomenon is probably a result of competition for active transport sites out of CSF between HVA and the increased quantities of 5-HIAA which has a high affinity for these sites (Moir & Yates, 1970). The concentration of 5-HIAA also rose slightly after saline and also after D-tryptophan but there was no significant difference between these two treatments. This rise was significantly lower ($P < 0.001$) than the rise seen after L-tryptophan. These results suggest that a rise in 5-HT synthesis occurs only after L-tryptophan. The small rise after saline or D-tryptophan is enigmatic but could be due either to non-specific stress (Thierry, Javoy, Glowinski & Kety, 1968) or to some impairment of the removal of 5-HIAA due to leakage of blood into the ventricular system when the blood proteins might interfere with transport mechanisms.

Our findings are clearly different from those of Yuwiler (1973). They suggest that little peripheral conversion from D-tryptophan to L-tryptophan occurs in the dog compared with the rat. The data also suggest that because of the similarities between the peripheral metabolism of D-tryptophan in man and dog that in looking at the cerebral metabolism of the D-isomer the dog is the more appropriate animal model than the rat.

Reprints from D.E. (MRC Brain Metabolism Unit).

## References

ASHCROFT, G.W., DOW, R.C. & MOIR, A.T.B. (1968). The active transport of 5-hydroxyindol-3-ylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid from a recirculatory perfusion system of the cerebral ventricles of the unanaesthetised dog. *J. Physiol., Lond.*, **199**, 397–425.

BALDWIN, H.R. & BERG, C.P. (1949). The influence of optical isomerism and acetylation upon the availability of tryptophan for maintenance in man. *J. Nutr.*, **39**, 203–218.

BERG, C.P. (1934). Tryptophane metabolism. IV the influence of optical activity on the utilization of tryptophane for growth and for kynurenic acid production. *J. biol. Chem.*, **104**, 373–384.

BERG, C.P. (1942). The metabolism of the d-amino acids. *Fedn Proc.*, **1**, 281

BERG, C.P. (1953). Physiology of the D-amino acids. *Physiol. Rev.*, **33**, 145–189.

CORRELL, J.T., BERG, C.P. & COWAN, D.W. (1938). Tryptophane metabolism. IX the excretion of kynurenic acid in the bile and urine of the dog after the administration of kynurenic acid and L- and D,L-tryptophane. *J. biol. Chem.*, **123**, 151–159.

ECCLESTON, D., ASHCROFT, G.W., MOIR, A.T.B., PARKER-RHODES, A., LUTZ, W. & O'MAHONEY, D.P. (1968). A comparison of 5-hydroxyindoles in various regions of dog brain and cerebrospinal fluid. *J. Neurochem.*, **15**, 947–957.

GAL, E.M., ARMSTRONG, J.C. & GINSBERG, B. (1966). The nature of *in vitro* hydroxylation of L-tryptophan by brain tissue. *J. Neurochem.*, **13**, 643–654.

GUROFF, G. & UDENFRIEND, S. (1962). Studies of aromatic amino acid uptake by rat brain *in vivo*. *J. biol. Chem.*, **237**, 803–806.

HANKES, L.V., BROWN, R.R., LEKLEM, J., SCHMAELER, M. & JESSEPH, J. (1972). Metabolism of $C^{14}$ labelled enantiomers of tryptophan kynurenine and hydroxykynurenine in humans with scleroderma. *J. Invest. Dermatol.*, **58**, 85–95.

HESS, S.M. & UDENFRIEND, S. (1959). A fluorometric procedure for the measurement of tryptamine in tissue. *J. Pharmac. exp. Ther.*, **127**, 175–177.

KENDALL, M.G. & STUART, A. (1967). *The Advanced Theory of Statistics*, Vol. II, pp. 346–373. London: Griffin.

McMENAMY, R.H. & ONCLY, J.L. (1958). The specific binding of L-tryptophan to serum albumin. *J. biol. Chem.*, **233**, 1436–1447.

MOIR, A.T.B. & YATES, C.M. (1970). Actions of phenelzine on the interactions of the metabolism of tryptophan and dopamine in brain. *Br. J. Pharmac.*, **40**, 563P.

OESTERLING, M.J. & ROSE, W.C. (1952). Tryptophan requirement for growth and utilization of its optical isomers. *J. biol. Chem.*, **196**, 33–43.

ROSE, W.C. (1949). Amino acid requirements of man. *Fedn. Proc.*, **8**, 546–552.

THIERRY, A.M., JAVOY, F., GLOWINSKI, J. & KETY, S.S. (1968). Effects of stress on the metabolism of norepinephrine, dopamine and serotonin in the central nervous system of the rat. *J. Pharmac. exp. Ther.*, **163**, 163–171.

VIGNEAUD, V. DU, SEALOCK, R.R. & VAN ETTEN, C. (1932). The availability of D-tryptophan and its acetyl derivative to the animal body. *J. biol. Chem.*, **98**, 565–575.

YATES, C.M. & GULDBERG, H.C. (1968). Some studies of the effects of chlorpromazine, reserpine and dihydroxyphenylalanine on the concentrations of homovanillic acid, 3,4-dihydroxyphenylacetic acid and 5-hydroxyindol-3-ylacetic acid in ventricular cerebrospinal fluid of the dog using the technique of serial sampling of the cerebrospinal fluid. *Br. J. Pharmac. Chemother.*, **33**, 457–471.

YUWILER, A. (1973). Conversion of D- and L-tryptophan to brain serotonin and 5-hydroxyindoleacetic acid and to blood serotonin. *J. Neurochem.*, **20**, 1099–1109.

*(Received March 5, 1976)*

*Br. J. Pharmac.* (1976), **58**, 9–16

# EFFECTS OF DRUGS AFFECTING ENDOGENOUS AMINES OR CYCLIC NUCLEOTIDES ON ETHANOL WITHDRAWAL HEAD TWITCHES IN MICE

H.O.J. COLLIER, M.D. HAMMOND & C. SCHNEIDER
Miles Laboratories Limited, Stoke Poges, Slough, SL2 4LY

**1** Twenty-four hours after ethanol withdrawal, dependent mice exhibited frequent head twitching. Naive mice exhibited similar twitching 15 min after treatment with 5-hydroxytryptophan (5-HTP) or 6 h after α-methyl-*p*-tyrosine (AMPT). Ethanol lessened the incidence of head twitches induced by any of these treatments. 5-HTP and AMPT each increased the incidence of head twitches induced by withdrawal of ethanol from dependent mice.

**2** Drugs that affect the amount or activity of endogenous amines or cyclic nucleotides modified the incidence of head twitches. Nearly all drugs acted in the same direction on twitching elicited by any of these three treatments.

**3** The incidence was lessened by: (a) methysergide, methergoline, MA 1420, *p*-chlorophenylalanine and *p*-chloroamphetamine; (b) dopamine, noradrenaline, L-DOPA, amphetamine and apomorphine; (c) hyoscine and nicotine; and (d) adenosine triphosphate, dibutyryl cyclic adenosine-3′,5′-monophosphate (db cyclic AMP) and prostaglandins $E_1$ and $E_2$.

**4** The incidence was increased by: (a) acetylcholine, carbachol and physostigmine; and (b) guanosine triphosphate, dibutyryl cyclic guanosine monophosphate (db cyclic GMP), theophylline and 3-isobutyl-1-methyl-xanthine.

**5** These findings suggest that head twitching induced by these three treatments arises from a common biochemical mechanism, which may ultimately be a change in favour of cyclic GMP of the balance between this nucleotide and cyclic AMP within appropriate neurones. This imbalance appears to be elicited or increased by 5-hydroxytryptamine and acetylcholine and to be decreased by dopamine, noradrenaline and E prostaglandins.

**6** Neither actinomycin D nor cycloheximide, given during the induction of ethanol dependence, altered the incidence of head twitches after ethanol withdrawal.

## Introduction

One of the obstacles to exploring the mechanism of ethanol dependence in experimental animals is a scarcity of recognizable abstinence signs. In mice, however, we observed that head twitching was much more frequent than usual between about 18 and 36 h after withdrawal of ethanol, administered subchronically either by mouth or by inhalation (Hammond & Schneider, 1973). For reasons discussed below, we regarded this as an abstinence sign. To explore the biochemical mechanism of ethanol dependence, we tested certain drugs, given after ethanol withdrawal, on the incidence of head twitching.

Because the intraperitoneal administration of 5-hydroxytryptophan (5-HTP), or the intracerebroventricular administration of 5-hydroxytryptamine (5-HT) had been shown to elicit head twitching in mice (Corne, Pickering & Warner, 1963; Mawson & Whittington, 1970), we began by testing drugs interacting with 5-HT mechanisms. The study was then extended to drugs affecting other brain amines (Collier, Hammond & Schneider, 1974). Because of the interaction of some amines with cyclic nucleotides (Weiss & Costa, 1968; Ferrendelli, Steiner, McDougal & Kipnis, 1970; Wellman & Schwabe, 1973; Daly, 1975) and because another drug of dependence, morphine, interacts with cyclic nucleotides (Collier & Roy, 1974; Gullis, Traber & Hamprecht, 1975), we then studied the effects of some other drugs affecting cyclic nucleotide mechanisms on the incidence of head twitches after ethanol withdrawal. These studies are described in detail below; some of these findings have been briefly reported (Hammond & Schneider, 1973; Collier *et al.*, 1974; Hammond & Schneider, 1974; Collier, Hammond & Schneider, 1976).

## Methods

### *Animal treatment and observation*

Male albino T/O strain mice weighing 23 to 25 g at the start of the treatment were used. They were randomized into groups of five and coloured for identification. In the ethanol withdrawal experiments, mice received by intragastric tube a daily dose of ethanol (40% w/v, in distilled water) starting at 4 g/kg (i.e. 10 ml/kg) on the first day and increasing daily by 1 g/kg to 7 g/kg on the fourth day. Head twitches were counted 24 h after the last dose of ethanol. In experiments in which head twitches were elicited in naive mice, 5-HTP (400 mg/kg in 20 ml saline/kg) was given intraperitoneally and head twitches were counted 15–19 min later. $\alpha$-Methyl-$p$-tyrosine (AMPT) (300 mg/kg suspended in 10% w/v gum acacia in distilled water) was given orally (10 ml/kg) and head twitches counted 6 h later.

Drugs tested for ability to modify the incidence of head twitches were given in a dose volume of 10 ml/kg by the oral, intraperitoneal or subcutaneous route. Intracerebroventricular injections (up to 50 µl in volume) were carried out by the method of Haley & McCormick (1957), modified by using 3 mm depth of injection and light ether anaesthesia. Most drugs were dissolved in 0.9% w/v NaCl solution (saline) or distilled water; MA 1420 was suspended in 10% w/v gum acacia in distilled water, and methergoline was dissolved in 1% w/v ascorbic acid in distilled water.

Individual head twitches were counted during periods of 4 min, after transferring the mice in groups of five to opaque plastic boxes (30 cm long × 12 cm high × 12 cm wide) fitted with a perforated Perspex lid. Observers did not know the treatment each mouse had received.

### *Design and analysis of experiments*

In experiments on the induction of head twitching, mice received either inducing treatment (ethanol, 5-HTP or AMPT) or vehicle. In experiments on the modification by drugs of the incidence of head twitches, some mice received inducing treatment, and, of these, some received modifying drug and the rest vehicle; other mice received both vehicles. The effect of a modifying drug on induced head twitching was expressed numerically as the ratio of the mean twitch count in mice treated with modifying drug to that in mice receiving its vehicle (the incidence ratio).

Because values were not distributed normally, statistical significance was determined by the non-parametric Mann-Whitney 'U' test (Siegel, 1956). When only one dose is reported, this is a submaximally effective dose. The experimental design did not allow statistical comparison to be made between drugs.

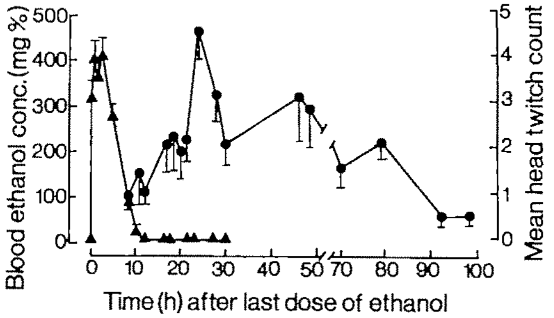


**Figure 1** Blood ethanol levels and incidence of head twitches in mice that had received oral doses of ethanol, increasing from 4 to 7 g/kg, once daily for four days. (▲) Ethanol concentration; (●) mean head twitch count/4 min; vertical lines represent s.e. mean.

Ethanol concentration in brain homogenate or blood was determined by gas-liquid chromatography, as described by Hammond (1975a).

### *Drugs*

The following drugs were used: acetylcholine hydrobromide, adenosine triphosphate (ATP), amphetamine sulphate, apomorphine hydrochloride, carbachol, dibutyryl cyclic-3′,5′-adenosine monophosphate (db cyclic AMP), dibutyryl cyclic-3′,5′-guanosine monophosphate (db cyclic GMP), L-DOPA, dopamine, ethanol (96% v/v), guanosine triphosphate (GTP), hyoscine hydrobromide, imidazole, 3-isobutyl-1-methylxanthine (IBMX), 6-acetamido-3-(3-(4-phenyl-1-piperazinyl)-propyl)-2,4-quinazolinedione maleate (MA 1420), methergoline, $\alpha$-methyl-$p$-tyrosine, methysergide bimaleate, nicotine hydrogen tartrate, noradrenaline hydrochloride, $p$-chlorophenylalanine methylester hydrochloride (PCPA), $p$-chloroamphetamine hydrochloride (PCA), physostigmine salicylate, prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$, theophylline. Doses are expressed in terms of active acid or base.

## Results

### *Induction of head twitching*

When ethanol was given once daily to mice on four successive days, its concentration in the blood reached a peak (400 mg %) 30–60 min after the last dose (Figure 1). The concentration then fell to $< 3$ mg % at 12 h after the last dose of ethanol. The concentration of ethanol in the brain closely paralleled that in blood.

As the blood ethanol concentration declined, head twitching increased to a peak, at which each mouse exhibited about 1 twitch/min, 24 h after the last dose.

**Table 1** Modification by ethanol and other drugs of the incidence of head twitches induced by ethanol withdrawal, by 5-hydroxytryptophan (5-HTP) or by $\alpha$-methyl-$p$-tyrosine (AMPT)

| *Modifying drug* | *Route* | *Dose* | *Incidence ratio after induction with* | | |
|---|---|---|---|---|---|
| | | | Ethanol | 5-HTP | AMPT |
| Ethanol | oral | 2000 mg | 1.70 | NT | NT |
| | | 4000 mg | 0.33† | 0.30† | 0.25† |
| | | 6000 mg | 0.11‡ | NT | NT |
| Methergoline | oral | 4 mg | 0.10‡ | 0.15‡ | 0.00† |
| Noradrenaline | i.c.v. | 5 µg | 0.25† | 0.40‡ | 0.25‡ |
| db cyclic AMP | i.c.v. | 100 µg | 0.10† | 0.35† | 0.05† |
| db cyclic GMP | i.c.v. | 50 µg | 2.28† | 3.20† | 1.75* |
| Theophylline | oral | 100 mg | 2.60† | 2.60‡ | 1.85† |
| Imidazole | oral | 100 mg | 1.15 | 0.20‡ | 1.10 |

Ethanol dependence was induced by oral doses of ethanol once daily for 4 days, increasing from 4 g/kg to 7 g/kg. Head twitches over a 4 min period were counted 24 h after the last dose of ethanol. 5-HTP (400 mg/kg) was given intraperitoneally (i.p.) 15 min before head twitch counting started. AMPT (300 mg/kg) was given orally 6 h before head twitch counting. Modifying drugs were given orally 1 h or i.c.v. 35 min before counting. Controls received the same inducing treatment as test animals, but only the vehicle of the modifying drug. The incidence ratio is the ratio of the head twitch count in mice receiving modifying drug to that in mice receiving vehicle of modifying drug.

NT, not tested; db cyclic AMP, dibutyryl cyclic AMP; db cyclic GMP, dibutyryl cyclic guanosine monophosphate. Oral doses in mg/kg; intracerebroventricular (i.c.v.) doses in µg/mouse.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ (Mann-Whitney 'U' Test).

**Table 2** Modification of the incidence of ethanol withdrawal head twitches by drugs affecting 5-hydroxytryptamine (5-HT) mechanisms

| *Effect sought* | *Modifying drug* | *Route* | *Dose (mg base/kg)* | *Incidence ratio* |
|---|---|---|---|---|
| Inhibition of 5-HT biosynthesis | PCPA | i.p. | 50 | 0.70 |
| | | | 200 | 0.63* |
| | PCA | i.p. | 1.25 | 0.74† |
| | | | 5 | 0.44† |
| | | | 20 | 0.35† |
| Specific antagonism of 5-HT | Methysergide | s.c. | 6 | 0.37‡ |
| | | | 24 | 0.13‡ |
| | Methergoline | s.c. | 0.4 | 0.44* |
| | | | 4 | 0.02* |
| | | oral | 4 | 0.10‡ |
| | MA 1420§ | oral | 25 | 0.71 |
| | | | 100 | 0.17‡ |
| Increase of 5-HT | 5-HTP | i.p. | 400 | 3.36* |

All mice received oral doses of ethanol, increasing from 4 to 7 g/kg, once daily for four days. $p$-Chlorophenylalanine (PCPA) and $p$-chloroamphetamine (PCA) were given intraperitoneally (i.p.) 1 h before each ethanol dose. Antagonists were given orally or subcutaneously (s.c.) 1 h, and 5-hydroxytryptophan (5-HTP) was given i.p. 15 min before counting started. Other details as Table 1.

§ Hong, 1973.

From this peak, head twitching slowly decreased during about 72 h to a normal level of about 0.15 twitch/min (Figure 1). Further treatment with ethanol suppressed the twitching in a dose-related way (Table 1). A comparable increase in head twitching may be observed when mice are withdrawn from continuous exposure for 8–14 days to 20 mg/l of ethanol in the air they breathe (Hammond, 1975b).

The head twitching in naive mice after treatment with 5-HTP or with AMPT was similar in appearance and incidence to that after ethanol withdrawal. Ethanol suppressed head twitches induced by 5-HTP or AMPT (Table 1), each of which in turn increased ethanol withdrawal twitching (Tables 2 and 3). Six of seven drugs tested modified head twitching induced by all three treatments in the same direction (Table 1). The seventh drug, imidazole, a weak stimulant of phosphodiesterase (Butcher & Sutherland, 1962) inhibited only the head twitching induced by 5-HTP.

*Modification by drugs of withdrawal head twitching*

Two inhibitors of protein biosynthesis were tested for their effect on the induction of ethanol withdrawal head twitching. Neither actinomycin D (50 µg/kg) nor cycloheximide (40 mg/kg), injected intraperitoneally 1 h before each daily dose of ethanol, significantly changed the incidence of head twitches, 24 h after ethanol withdrawal.

*Drugs affecting 5-hydroxytryptamine mechanisms.* With two exceptions, drugs tested for ability to modify the expression of withdrawal head twitching were given after the last dose of ethanol. When given during the induction of ethanol dependence, the inhibitors of 5-HT biosynthesis, PCPA and PCA, significantly reduced the incidence of head twitches after ethanol withdrawal, PCA being roughly 40 times more potent than PCPA (Table 2). Because, in this instance, the effect might have resulted from an inhibition of the induction of ethanol dependence, three specific antagonists of 5-HT were tested, by giving each in a single dose 23 h after the last dose of ethanol. All these antagonists reduced the incidence of withdrawal head twitching, whereas 5-HTP increased its incidence (Table 2).

**Table 3** Modification of the incidence of ethanol withdrawal head twitches by drugs affecting catecholamine mechanisms

| *Effect sought* | *Modifying drug* | *Route* | *Dose* | *Incidence ratio* |
|---|---|---|---|---|
| Increase of brain dopamine | L-DOPA | oral | 2 × 50 mg | 1.06 |
| | | | 2 × 200 mg | 0.51† |
| | | | 2 × 800 mg | 0.64† |
| | Dopamine | i.c.v. | 50 µg | 0.31* |
| | | | 150 µg | 0.04‡ |
| | | i.v. | 1000 µg | 0.84 |
| Stimulation of dopamine receptor | Apomorphine | oral | 1 mg | 0.47* |
| | | | 5 mg | 0.66* |
| Increase of brain noradrenaline | Noradrenaline | i.c.v. | 2.5 µg | 0.27† |
| | | | 5 µg | 0.25† |
| | | | 10 µg | 0.11‡ |
| | | i.v. | 10 µg | 1.23 |
| | Amphetamine | s.c. | 5 mg | 0.58* |
| | | | 10 mg | 0.23‡ |
| | | | 20 mg | 0.00‡ |
| Inhibition of catecholamine biosynthesis | AMPT | oral | 300 mg | 2.00† |

L-DOPA was given in two doses, one at 18 h and one at 1 h before counting head twitches. Dopamine and noradrenaline were given 35 min before counting. Other drugs were given 1 h before counting. Oral and subcutaneous (s.c.) doses are in mg/kg; intracerebroventricular (i.c.v.) and intravenous (i.v.) doses in µg/mouse. Other details as in Tables 1 and 2.

*Drugs affecting catecholamine mechanisms.* Dopamine or noradrenaline inhibited ethanol withdrawal head twitching. Whereas a single intracerebroventricular injection of either catecholamine was highly effective, intravenous injection was not (Table 3). L-DOPA was, however, effective when given in two oral doses after ethanol withdrawal. Other drugs that had sympathomimetic activity, such as apomorphine and amphetamine, also lessened withdrawal head twitching; whereas AMPT, which inhibits endogenous biosynthesis of dopamine and noradrenaline (Spector, Sjoerdsma & Udenfriend, 1965), increased it.

*Drugs affecting acetylcholine mechanisms.* By the intracerebroventricular route, acetylcholine or carbachol increased the incidence of ethanol withdrawal head twitching whereas nicotine reduced it (Table 4). By the intraperitoneal route, physostigmine increased head twitching and hyoscine lessened its incidence.

*Cyclic nucleotides.* By the intracerebroventricular route, db cyclic AMP lessened considerably the incidence of withdrawal head twitches, whereas db cyclic GMP increased their incidence (Table 5). After db cyclic AMP, mice were slightly sedated. ATP and GTP given intraperitoneally, affected the incidence of twitches in the same direction as did the dibutyryl salts of the corresponding cyclic nucleotides. The phosphodiesterase inhibitors, theophylline and IBMX, increased withdrawal head twitching; but imidazole was inactive (Table 5). Prostaglandins $E_1$ and $E_2$, which increase brain cyclic AMP, also lessened head twitching but prostaglandin $F_{2\alpha}$, which has little effect on brain cyclic AMP, was inactive.

If theophylline and IBMX acted by inhibiting phosphodiesterase, they would be expected to increase neuronal cyclic AMP, which probably inhibits head twitching, as well as cyclic GMP, which probably intensifies it. To investigate why theophylline and IBMX increased head twitching, we tested the effect of intracerebroventricular injections, at a dose of 50 µg/mouse, of mixtures of db cyclic AMP and db cyclic GMP, 20 min before intraperitoneal injection of 5-HTP (400 mg/kg). Compared with saline, a mixture of 25 µg db cyclic AMP and 25 µg db cyclic GMP increased the incidence ratio due to 5-HTP to 2.34 ($P < 0.05$). Even as little as 5 µg db cyclic GMP in the mixture increased this ratio to 1.69. Hence an increase of cyclic GMP would outweigh an increase of cyclic AMP in their effects on head twitching.

## Discussion

Three findings argue that the large increase of head twitching that occurs after withdrawal of ethanol, given subchronically to mice, is an abstinence sign. First, increased head twitching was not seen at any time after a single intragastric dose of 4 g/kg ethanol; but it was seen after this dose had been repeated on three successive days. Second, after withdrawal of ethanol, head twitching increases as the blood and brain concentrations of ethanol fall and it reaches a peak when ethanol can no longer be detected. Third, increased head twitching is suppressed by giving a large dose of ethanol.

Several findings suggest that the increased head twitching elicited by ethanol withdrawal, 5-HTP and AMPT arises centrally. Thus, twitching elicited by these treatments was intensified by db cyclic GMP and inhibited by noradrenaline or db cyclic AMP, injected by the intracerebroventricular route (Table 1). Again, ethanol withdrawal head twitching, although

**Table 4** Modification of the incidence of ethanol withdrawal head twitches by drugs affecting acetylcholine (ACh) mechanisms

| *Effect sought* | *Modifying drug* | *Route* | *Dose* | *Incidence ratio* |
|---|---|---|---|---|
| Increase of brain ACh | Acetylcholine | i.c.v. | 10 µg | 1.56* |
| | Physostigmine | i.p. | 0.02 mg | 1.63* |
| Simulation of ACh | Carbachol | i.c.v. | 1 µg | 1.81* |
| | Nicotine | i.c.v. | 0.125 µg | 0.22 |
| | | | 0.5 µg | 0.20* |
| | | | 1 µg | 0.00† |
| Specific antagonism of ACh | Hyoscine | i.p. | 2 mg | 0.33* |

Acetylcholine, carbachol and nicotine were given 35 min and physostigmine and hyoscine 1 h before counting head twitches began. Intraperitoneal (i.p.) doses are in mg/kg; intracerebroventricular (i.c.v.) doses in µg/mouse. Other details as in Tables 1 and 2.

inhibited by intracerebroventricular dopamine or noradrenaline, was not inhibited by larger intravenous doses of either drug (Table 3). It was, moreover, intensified by intracerebroventricular acetylcholine or carbachol, but lessened by intracerebroventricular nicotine (Table 4). Yet again, 5-HT elicits head twitching when injected by the intracerebroventricular route (Mawson & Whittington, 1970).

Tables 2–5 give the effects sought by administering modifying drugs to mice dependent on ethanol. It is not contended that these are the only effects of the drugs given, but there is good evidence in the literature that they are the outstanding effects. The internal consistency of the results obtained with drugs modifying each mechanism in different ways supports the validity of this approach.

A seeming exception is nicotine, which lessens head twitching, whereas acetylcholine, physostigmine and carbachol increase it. This exception is, however, consistent with the findings that stimulation of nicotine receptors in rat adrenal medulla increases cyclic AMP formation (Guidotti & Costa, 1974) and that stimulation of muscarinic but not nicotinic receptors in chick cerebral hemispheres increases the level of cyclic GMP (Nahorski, Pratt & Rogers, 1976).

The main aim of this work was to elucidate the biochemical mechanism of ethanol dependence by studying the effect on an abstinence sign of drugs modifying brain amine and/or cyclic nucleotide mechanisms. The results are unexpectedly clear-cut. On the one hand, drugs that probably increase the level or activity of 5-HT, acetylcholine or cyclic GMP intensify head twitching, and drugs that probably decrease any of these inhibit head twitching. On the other hand, drugs that probably increase the level or activity of dopamine, noradrenaline or cyclic AMP inhibit head twitching and drugs that probably decrease any of these intensify it. Hence it may be supposed that head twitching arises from a change in favour of cyclic GMP of the balance between this nucleotide and cyclic AMP, resulting from a change in the balance of neurohumoral mediators, particularly against dopamine and/or noradrenaline.

There is evidence from the experiments of Carlsson and colleagues with AMPT (Carlsson, Engel & Svensson, 1972; Ahlenius, Carlsson, Engel, Svensson & Södersten, 1973; Engel, Strömbom, Svensson &

**Table 5** Modification of the incidence of ethanol withdrawal head twitches by drugs related to cyclic nucleotides

| *Effect sought* | *Modifying drug* | *Route* | *Dose* | *Incidence ratio* |
|---|---|---|---|---|
| Increase of brain cyclic AMP | db cyclic AMP | i.c.v. | 50 µg | 0.95 |
| | | | 100 µg | 0.10† |
| | ATP | i.p. | 50 mg | 0.36† |
| Stimulation of adenylate cyclase | Prostaglandin $E_1$ | s.c. | 0.125 mg | 0.38 |
| | | | 0.5 mg | 0.21‡ |
| | | | 2 mg | 0.03‡ |
| | Prostaglandin $E_2$ | s.c. | 0.5 mg | 0.41* |
| | Prostaglandin $F_{2\alpha}$ | s.c. | 2 mg | 0.86 |
| Increase of brain cyclic GMP | db cyclic GMP | i.c.v. | 50 µg | 2.28† |
| | GTP | i.p. | 20 mg | 2.07† |
| Inhibition of phosphodiesterase§ | Theophylline | oral | 100 mg | 2.60† |
| | IBMX | oral | 10 mg | 2.11† |
| Stimulation of phosphodiesterase | Imidazole | oral | 100 mg | 1.15 |

Dibutyryl cyclic AMP (db cyclic AMP) and dibutyryl cyclic 3′,5′-guanosine monophosphate (db cyclic GMP) were given 35 min before counting head twitches began. ATP and guanosine triphosphate (GTP) were given 19 h before counting. The remaining drugs were given 1 h before counting. Other details as in Tables 1 and 2.
§ Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970.

Waldeck, 1974) that ethanol produces euphoria and other effects by stimulating appropriate brain neurones responding to catecholamines. There is also evidence from our experiments and from those of Goldstein (1973), Blum & Wallace (1974) and Griffiths, Littleton & Ortiz (1974) that abstinence effects are associated with a relative lowering of the amount or activity of catecholamines in the brain during ethanol withdrawal. The steps by which continued treatment with ethanol produces a state of dependence may be surmised on the basis of published evidence to be as follows. First, ethanol liberates catecholamines in the brain, thus stimulating neurones concerned with its pharmacological effects and increasing catecholamine turnover (Thadani & Truitt, 1973; Hunt & Majchrowicz, 1974; Pohorecky, 1974). Second, probably as a result of this effect, there is a slow increase of dopamine and noradrenaline concentrations in the brain during continued treatment with ethanol, followed by a rapid decline after its withdrawal (Griffiths *et al.,* 1974). Third, an increase in catecholamine concentration at the specific receptors causes a decrease in the number of these receptors, as observed in other situations (Mukherjee, Caron & Lefkowitz, 1975; Mickey, Tate & Lefkowitz, 1975; Kebabian, Zatz, Romero & Axelrod, 1975). Fourth, such a decrease in receptor numbers leads to subsensitivity towards catecholamines in the stimulation of neuronal cyclic AMP formation, as observed in slices of the cerebral cortex of the rat chronically treated with ethanol (French, Palmer, Narod, Reid & Ramey, 1975). Fifth, on withdrawal of ethanol, a shortage of catecholamines and/or a subsensitivity towards them produces the imbalance that leads to abstinence effects, as described above. This conjectural mechanism of dependence, based on the development of subsensitivity to a neurohumoral transmitter through a drug-induced change in receptor number, conforms with the hypothesis proposed some years ago (Collier, 1966). A comparable mechanism involving supersensitivity to 5-HT could be envisaged. The earlier steps in this mechanism would also give rise to tolerance.

The evidence of our experiments and that in the literature summarized above suggests that L-DOPA may be useful in the clinical management of alcoholism. It is possible, too, that specific antagonists of 5-HT might also be useful in this condition.

The authors wish to thank Dr A.F. Green of the Wellcome Research Laboratories for the gift of methergoline used in this study. We are also grateful to Mr N.J. Cuthbert, Miss J. Czogala and Miss E.A. Wilson for their help in observing drug effects and to Mr L.C. Dinneen for statistical advice.

## References

AHLENIUS, S., CARLSSON, A., ENGEL, J., SVENSSON, T. & SÖDERSTEN, P. (1973). Antagonism by alpha methyltyrosine of the ethanol-induced stimulation and euphoria in man. *Clin. Pharmac. Ther.,* **14,** 586–591.

BEAVO, J.A., ROGERS, N.L., CROFFORD, C.B., HARDMAN, J.G., SUTHERLAND, E.W. & NEWMAN, E.V. (1970). Effects of xanthine derivatives on lipolysis and on adenosine 3′,5′-monophosphate phosphodiesterase activity. *Molec. Pharmac.,* **6,** 597–603.

BLUM, J. & WALLACE, J.E. (1974). Effects of catecholamine synthesis inhibition on ethanol-induced withdrawal symptoms in mice. *Br. J. Pharmac.,* **51,** 109–111.

BUTCHER, R.W. & SUTHERLAND, E.W. (1962). Adenosine 3′,5′-phosphate in biological materials. *J. biol. Chem.,* **237,** 1244–1250.

CARLSSON, A., ENGEL, J. & SVENSSON, T. (1972). Inhibition of ethanol-induced excitation in mice and rats by α-methyl-*p*-tyrosine. *Psychopharmacologia (Berl.),* **26,** 307–312.

COLLIER, H.O.J. (1966). Tolerance, physical dependence and receptors. *Adv. Drug Res.,* **3,** 171–188.

COLLIER, H.O.J., HAMMOND, M.D. & SCHNEIDER, C. (1974). Biogenic amines and head twitches in mice during ethanol withdrawal. *Br. J. Pharmac.,* **51,** 310–311.

COLLIER, H.O.J., HAMMOND, M.D. & SCHNEIDER, C. (1976). Neuro-humoral transmitters, cyclic nucleotides and experimental dependence on ethanol in the mouse. Symposium Franco-Britannique sur l'alcoolisme. *Les Colloques de l'Institut National de la Santé et de la Recherche Médicale, Paris,* **54,** 161–164.

COLLIER, H.O.J. & ROY, A.C. (1974). Morphine-like drugs inhibit the stimulation by E prostaglandins of cyclic AMP formation by rat brain homogenate. *Nature, Lond.,* **248,** 24–27.

CORNE, S.J., PICKERING, R.W. & WARNER, B.T. (1963). A method for assessing the effects of drugs on the central actions of 5-hydroxytryptamine. *Br. J. Pharmac.,* **20,** 106–120.

DALY, J.W. (1975). Cyclic adenosine 3′,5′-monophosphate role in the physiology and pharmacology of the central nervous system. *Biochem. Pharmac.,* **24,** 159–164.

ENGEL, J., STRÖMBOM, U., SVENSSON, T.H. & WALDECK, B. (1974). Suppression by α-methyl-tyrosine of the ethanol-induced locomotor stimulation: partial reversal by L-DOPA. *Psychopharmacologia (Berl.),* **37,** 275–279.

FERRENDELLI, J.A., STEINER, A.L., McDOUGAL, D.B. & KIPNIS, D.N. (1970). The effect of oxotremorine and atropine on cGMP and cAMP levels in mouse cerebral cortex and cerebellum. *Biochem. Biophys. Res. Comm.,* **41,** 1061–1067.

FRENCH, S.W., PALMER, D.S., NAROD, M.E., REID, P.E. & RAMEY, C.W. (1975). Noradrenergic sensitivity of the cerebral cortex after chronic ethanol ingestion and withdrawal. *J. Pharmac. exp. Ther.,* **194,** 319–326.

GOLDSTEIN, D.B. (1973). Alcohol withdrawal reactions in mice: effects of drugs that modify neurotransmission. *J. Pharmac. exp. Ther.,* **186,** 1–9.

GRIFFITHS, P.J., LITTLETON, J.M. & ORTIZ, A. (1974). Changes in monoamine concentrations in mouse brain associated with ethanol dependence and withdrawal. *Br. J. Pharmac.,* **50,** 489–498.

GUIDOTTI, A. & COSTA, E. (1974). A role for nicotinic receptors in the regulation of the adenylate cyclase of adrenal medulla. *J. Pharmac. exp. Ther.*, **189**, 665–675.

GULLIS, R., TRABER, J. & HAMPRECHT, B. (1975). Morphine elevates levels of cyclic GMP in a neuroblastoma × glioma hybrid cell line. *Nature, Lond.*, **256**, 57–59.

HALEY, T.J. & McCORMICK, W.G. (1957). Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br. J. Pharmac. Chemother.*, **12**, 12–15.

HAMMOND, M.D. (1975a). The use of an internal standard in the determination of ethanol in blood, brain and vapour. *Br. J. Addict.*, **70**, 162–164.

HAMMOND, M.D. (1975b). Pharmacological and Behavioural Investigation of Models of Alcoholism in Rodents. Ph.D. thesis, Chelsea College, University of London.

HAMMOND, M.D. & SCHNEIDER, C. (1973). Behavioural changes induced in mice following termination of ethanol administration. *Br. J. Pharmac.*, **47**, 667P.

HAMMOND, M.D. & SCHNEIDER, C. (1974). Cyclic nucleotides and ethanol withdrawal head twitches in mice. *Br. J. Pharmac.*, **52**, 138P.

HONG, E. (1973). On the antiserotonin activity of 6-acetamido-3-(3-(4-phenyl-1-piperazinyl)-propyl) 2, 8, 4 (1h, 2h)-quinazolinedione maleate (MA 1420). *Arzneim. Forsch.*, **23**, 1726–1728.

HUNT, W.A. & MAJCHROWICZ, E. (1974). Alterations in the turnover of brain norepinephrine and dopamine in ethanol dependent rats. *J. Neurochem.*, **23**, 549–552.

KEBABIAN, J.W., ZATZ, M., ROMERO, J.A. & AXELROD, J. (1975). Rapid changes in rat pineal $\beta$-adrenergic receptor: alterations in $^3$H-(1)-alprenolol binding and adenylate cyclase. *Proc. Nat. Acad. Sci. U.S.A.*, **72**, 3735–3739.

MAWSON, C. & WHITTINGTON, H. (1970). Evaluation of the peripheral and central antagonist activity against 5-hydroxytryptamine of some new agents. *Br. J. Pharmac.*, **39**, 223P.

MICKEY, J., TATE, R. & LEFKOWITZ, R.J. (1975). Subsensitivity of adenylate cyclase and decreased $\beta$-adrenergic receptor binding after chronic exposure to (–)-Isoproterenol *in vitro*. *J. biol. Chem.*, **250**, 5727–5729.

MUKERJEE, C., CARON, M.G. & LEFKOWITZ, R.J. (1975). Catecholamine-induced subsensitivity of adenylate cyclase associated with loss of $\beta$-adrenergic receptor binding sites. *Proc. Nat. Acad. Sci. U.S.A.*, **72**, 1945–1949.

NAHORSKI, S.R., PRATT, C.N.F.W. & ROGERS, K.J. (1976). Increased cerebral cyclic GMP concentration induced by muscarinic cholinergic agonists and prostaglandin $F_{2\alpha}$. *Br. J. Pharmac.*, (in press).

POHORECKY, L.A. (1974). Effect of ethanol on central and peripheral noradrenergic neurones. *J. Pharmac. exp. Ther.*, **189**, 380–391.

SIEGEL, S. (1956). *Nonparametric statistics for the behavioural sciences.* pp. 116–127. New York: McGraw-Hill.

SPECTOR, S., SJOERDSMA, A. & UDENFRIEND, S. (1965). Blockade of endogenous norepinephrine synthesis by $\alpha$-methyltyrosine, an inhibitor of tyrosine hydroxylase. *J. Pharmac. exp. Ther.*, **147**, 86–95.

THADANI, P.V. & TRUITT, E.G.L. (1973). Norepinephrine turnover effects of ethanol and acetaldehyde in rat brain. *Fedn. Proc.*, **32**, 697.

WEISS, B. & COSTA, E. (1968). Selective stimulation of adenyl cyclase of rat pineal gland by pharmacologically active catecholamines. *J. Pharmac. exp. Ther.*, **161**, 316–319.

WELLMAN, W. & SCHWABE, U. (1973). Effects of prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ on cyclic AMP levels in brain *in vivo*. *Brain Res.*, **59**, 371–378.

*(Received June 2, 1975.*
*Revised April 15, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 17–25

# FURTHER STUDIES ON THE MODE OF ACTION OF ISOPRENALINE ON GASTRIC SECRETION IN THE CONSCIOUS RAT

L. LUNDELL & S.E. SVENSSON
Department of Physiology, University of Lund, Lund, Sweden
G. NILSSON
Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

**1** The effect of isoprenaline on gastric secretion evoked by various means has been studied in conscious rats provided with Pavlov and Heidenhain pouches.

**2** Interdigestive acid secretion in the Pavlov pouch was reduced by isoprenaline, whereas pepsin secretion was unaltered.

**3** Central vagal stimulation effected by 2-deoxy-D-glucose injection evoked a gastric secretory response that was substantially reduced by isoprenaline.

**4** 2-Deoxy-D-glucose increased the mobilization of gastric mucosal histamine, an effect that was prevented by isoprenaline.

**5** Isoprenaline infusion alone induced a slight increase in histamine mobilization and also a considerable elevation of immunoreactive serum gastrin concentration.

**6** The secretory response to food in the Pavlov pouch was almost abolished by isoprenaline.

**7** Although the acid response to histamine in the Heidenhain pouch was susceptible to isoprenaline inhibition, that to methacholine was not.

**8** Pepsin secretion in the Heidenhain pouch preparation stimulated by histamine or methacholine seemed to be enhanced by isoprenaline.

## Introduction

In the rat, mobilization of gastric mucosal histamine may be closely related to the excitation of the parietal cell. Gastrin mobilizes histamine in a dose-dependent way from cells in close proximity to the parietal cells, in amounts sufficient to stimulate hydrochloric acid secretion (Kahlson, Rosengren, Svahn & Thunberg, 1964; Johansson, Lundell, Rosengren & Svensson, 1972; Lundell, 1974a, b). Consequently, the mode of action of gastrin in stimulating HCl secretion has been suggested to be by means of histamine mobilization. This suggestion has been further strengthened by the results obtained with histamine $H_2$-receptor antagonists on gastrin-induced secretion (Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973; Lundell, 1973; 1975a).

It has recently been demonstrated in this laboratory that isoprenaline inhibits pentagastrin-induced acid secretion in the rat and also restrains the increase in mucosal histamine mobilization, otherwise seen on pentagastrin infusion (Lundell & Svensson, 1974). These effects of isoprenaline were almost completely prevented by propranolol, indicating involvement of a $\beta$-adrenoceptor.

In the present study, the effect of isoprenaline was investigated on vagally-induced gastric secretion and histamine mobilization. Although vagus nerve stimulation is followed by both an increased mobilization of the amine and a direct activation of the parietal cells, the role of mobilized histamine for the resulting secretory response is far from settled (Rosengren & Svensson, 1969; Hakanson & Liedberg, 1970; Johansson *et al.*, 1972; Lundell, 1974b; 1975a). Furthermore, the effect of isoprenaline was studied on histamine and methacholine-induced gastric secretion, since these agents are considered to excite acid secretion without alterations in the mobilization of mucosal histamine. In order to obtain further information on the mode of action of isoprenaline, the serum concentrations of gastrin and glucose were determined in some relevant conditions.

## Methods

### *Determination of gastric secretion*

Female rats of the Sprague-Dawley strain, weighing about 250 g, and fed on a standard pellet diet, were used. All operations were done under ether anaesthesia. The rats were provided with Heidenhain (vagally denervated) pouches in the main according to Alphin & Lin (1959) or Pavlov (innervated) pouches as described by Svensson (1970). At least one month was allowed for postoperative recovery. Rats were fasted for 18 h before experiments except when the secretory response to food was studied when the fasting period was 24 hours. During the experiments the rats were kept unanaesthetized in Bollman cages. Gastric juice was collected in 30 min samples by the use of a perfusion technique (Svensson, 1970) and the amount of HCl secreted was determined by titration against 0.1 M NaOH with phenol red as an indicator. The pepsin output was measured by a modification of the method of Hunt (1948). The amount of HCl is expressed in µEq/30 min and pepsin output in µg/30 min, with the corresponding activity of a commercial crystalline preparation of pepsin (Lot 95B – 1270, Sigma Chemical Co) used as a standard as proposed by Bitsch (1966). Throughout an experiment, a constant infusion of 0.9% w/v NaCl solution (saline), into a catheter inserted in a tail or neck vein, was maintained by a motor-driven syringe. After interdigestive secretion had been collected for 1–2 h, drugs were added to the infusate. In the feeding experiments, food was allowed for 30 min, when the rats consumed 2–3 g of the standard pellet diet. Isoprenaline infusion did not apparently influence the amount of food ingested.

The following drugs were used: methacholine chloride (Fluka AG), 2-deoxy-D-glucose (Sigma Chemical Co), (±)-isoprenaline sulphate, (±)-propranolol hydrochloride (Inderal, ICI) and histamine dihydrochloride (E. Merck AG – Darmstadt). The doses of all agents are expressed in terms of the salt, except for histamine which is given in terms of the base.

### *Determinations of content and formation of mucosal histamine*

Observations were made on intact rats fasted for 18 h and injected intravenously with 2-deoxy-D-glucose alone or in combination with either isoprenaline or isoprenaline plus propranolol. The effect of isoprenaline infusion alone was also studied. The animals were killed by a blow on the head and exsanguinated. The stomach was opened along the lesser curvature, washed with saline, pinned flat and blotted with gauze; the oxyntic gland area was removed by scraping with a scalpel. After mincing, the mucosa from one stomach was divided in two portions, one of which was used for determinations of histamine content, the other for formation. Histamine content was determined by a modification of the method of Feldberg & Talesnik (1953) as described in detail by Lundell (1974a).

The rate of histamine formation, i.e. histidine decarboxylase activity, was determined by a method devised by Kobayashi (1963), as adapted for use in this laboratory (Henningsson, Lundell & Rosengren, 1974). The minced mucosa was incubated for 3 h at 37°C under $N_2$ in beakers containing 100 mg tissue, 40 µg [$^{14}$C-carboxyl]-L-histidine (sp. act. 104.9 µCi/mmol), 1 mM disodium edetate (EDTA), 0.5 mM glutathione, 10 µM pyridoxal-5-phosphate, 0.1M sodium phosphate buffer pH 7.4 and 0.2% w/v glucose, the total volume made up to 3 ml. In the blanks 10 mM semicarbazide was added. The incubation was stopped by gentle tipping of 1 ml 2 M citric acid from a side arm into the incubation mixture. Adequate $^{14}CO_2$ trapping into the Hyamine 10–x (0.1 mmol) on the filter paper, placed within the incubation vessel, was achieved by continuing mechanical shaking for another 45 minutes. The filter paper was placed in a counting vessel containing 10 ml of Bray (1960) scintillation solution and counted in a liquid scintillation spectrometer.

### *Determinations of serum gastrin*

Serum gastrin was determined in intact rats fasted for 18 hours. A polyethylene tube was inserted in a tail vein for administration of isoprenaline alone or combined with propranolol. For collection of blood, another polyethylene tube was inserted in the external jugular vein and the tip was passed into the superior caval vein. These procedures required ether anaesthesia for about 10 min, after which a period of at least 3 h was allowed for recovery. The tube for collection of blood was kept patent by intermittent flushing with a heparin solution (10 iu/ml). During the experiment, blood was drawn at 30 min intervals (usually 0.5 ml). After centrifugation, serum was decanted and stored at −20°C until analysis. The immunoreactive gastrin concentration was determined by a specific radioimmunoassay (Nilsson, 1975). The antiserum employed in the present study (2604-8; Rehfeld, Stadil & Vikelsøe, 1974) was used in a final dilution of 1:120.000. Mono-iodinated $^{125}$I synthetic human gastrin I was employed as tracer. To elevate the extent to which the assay measured the amount of the hormone in the actual species, rat serum was diluted and tested over a 10-fold range of concentration. The immunoreactive rat gastrin could not be distinguished from the porcine gastrin I standard. However, since rat gastrin was not used as a reference standard, the values relate to porcine gastrin I standard.

### *Determinations of serum glucose*

Blood was drawn from an external jugular vein at 30 min intervals. Saline and isoprenaline were infused into a tail vein. Serum was decanted and glucose con-

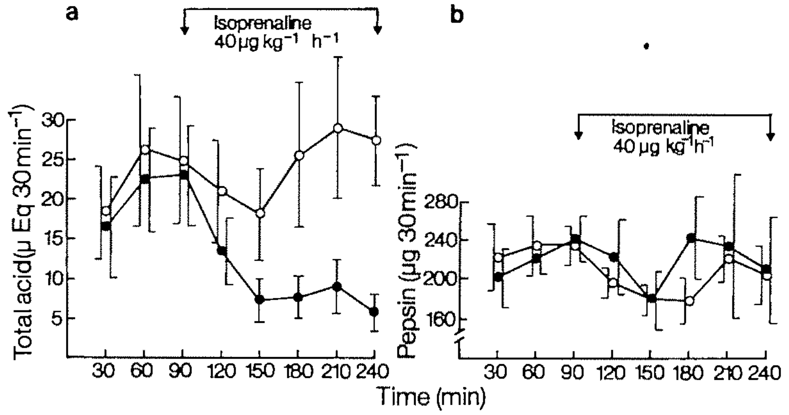


**Figure 1** Effect of Isoprenaline infusion (40 µg kg⁻¹ h⁻¹) on interdigestive secretion (●) of (a) acid and (b) pepsin. Each point represents the mean of one determination in each of six Pavlov pouch rats. The vertical lines show s.e. mean. (O) = Control values.

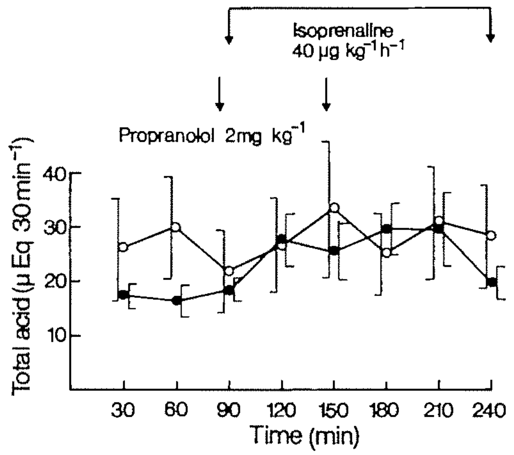


**Figure 2** Effect of combined intravenous administration of isoprenaline (40 µg kg⁻¹ h⁻¹) and propranolol (2 mg/kg) on interdigestive acid secretion (●). Propranolol was given as indicated by the arrows. Each point represents the mean of one determination in each of six Pavlov pouch rats. The vertical lines show s.e. mean. 'O) = Control values.

centration determined by the glucose-oxidase technique.

*P*-values were obtained by using Student's *t*-test for paired or unpaired values.

## Results

### *The effect of isoprenaline on interdigestive secretion*

Interdigestive acid and pepsin secretion (i.e. during saline infusion) were investigated in six Pavlov pouch rats for 4 hours. Interdigestive secretion of acid was about 25 µEq/30 min and that of pepsin about 220 µg/30 minutes. After 1 h of isoprenaline infusion (40 µg kg⁻¹ h⁻¹) acid secretion was reduced to a value of about 30% of the control level whereas the pepsin output was unaltered (Figure 1a and b). The pronounced effect os the $\beta$-adrenoceptor agonist on interdigestive acid secretion was completely abolished by propranolol (2 mg/kg) (Figure 2).

### *Inhibition by isoprenaline of the secretory response to 2-deoxy-D-glucose*

Central vagal excitation effected by 2-deoxy-D-glucose injection (100 mg/kg) was followed by a rapid increase in acid secretion with a peak of 83.3 ± 25.80 (s.e. mean) µEq/30 min after 60 min (Figure 3a). Isoprenaline infusion (40 µg kg⁻¹ h⁻¹) substantially reduced the response to 2-deoxy-D-glucose, attaining a value of 34.7 ± 11.32 µEq/30 min after 60 minutes. This reduction is highly significant ($P < 0.01$). Propranolol completely antagonized the effect of isoprenaline.

Pepsin secretion in response to 2-deoxy-D-glucose rose rapidly to a peak of 654 ± 80.9 µg/30 min after 60 min after which the secretory rate diminished (Figure 3b). Isoprenaline reduced the peak response to a value about 55% less than with 2-deoxy-D-glucose alone. Propranolol also blocked this inhibitory effect of isoprenaline (Figure 3b). In another series of experiments, isoprenaline infusion (40 µg kg⁻¹ h⁻¹) started 1 h before injection of 2-deoxy-D-glucose (100 mg/kg). The effect of isoprenaline was the same as described above. A background infusion of histamine (1000 µg/h), which started 30 min before 2-deoxy-D-glucose injection did not significantly diminish the inhibitory effect of isoprenaline.

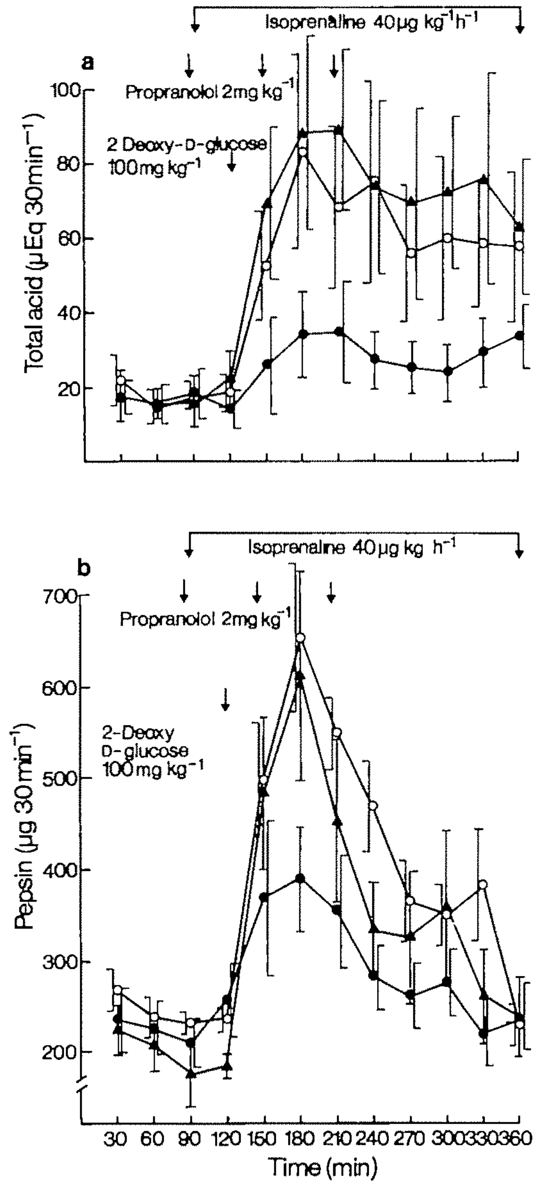


**Figure 3** (a) The acid and (b) pepsin secretory effects of 2-deoxy-D-glucose (100 mg/kg) injection alone (○) or combined with isoprenaline (40 µg kg⁻¹ h⁻¹) (●). Included is the effect of 2-deoxy-D-glucose combined with both isoprenaline and propranolol (2 mg/kg) (▲). Propranolol was given as indicated by the arrows. Each point represents the mean of one determination in each of six Pavlov pouch rats. The vertical lines show s.e. mean.

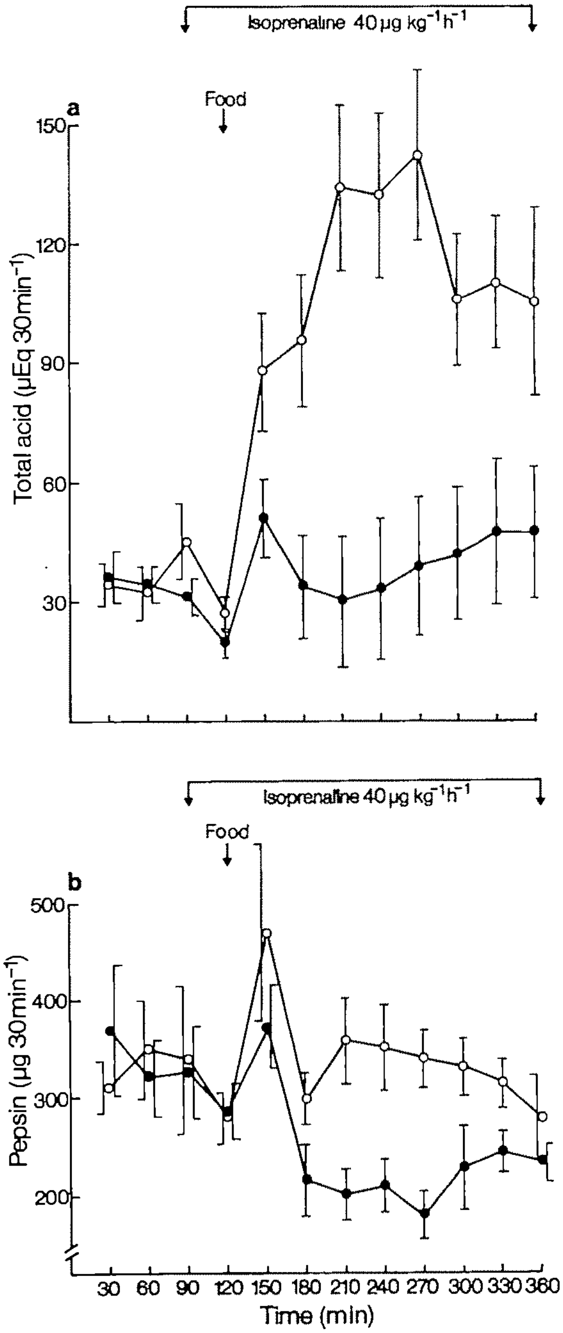


**Figure 4** (a) Acid and (b) pepsin secretion in response to food alone (○) or combined with isoprenaline infusion (40 µg kg⁻¹ h⁻¹) (●). Each point represents the mean of one determination in each of six Pavlov pouch rats. The vertical lines show s.e. mean.

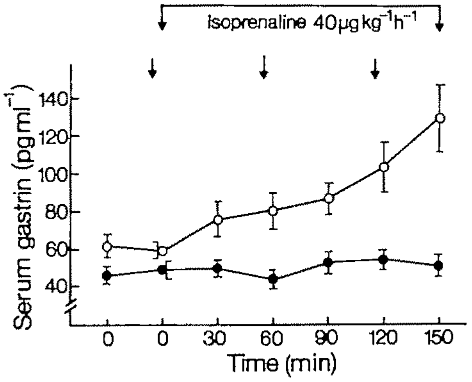


**Figure 5** Serum gastrin level (pg ml$^{-1}$) during isoprenaline infusion (40 µg kg$^{-1}$ h$^{-1}$) alone (○) or combined with propranolol (2 mg kg$^{-1}$) injection (●). Propranolol was injected as indicated by the arrows. Each point represents the mean of one determination in each of six rats. The vertical lines show s.e. mean.

*Isoprenaline inhibition of the feeding response*

Isoprenaline infusion almost abolished the response to feeding in six Pavlov pouch rats and only an initial response of about 50 µEq/30 min was recorded (Figure 4a). Feeding induced a pepsin secretory response that lasted only for 30 minutes. During isoprenaline infusion, a small initial pepsin response was noted, after which the response was significantly lower than in the controls ($P < 0.01$) (Figure 4b).

*Inhibition of the mobilization of mucosal histamine by isoprenaline*

Histamine mobilization was estimated by concomitant determination of mucosal histamine content and formation. 2-Deoxy-D-glucose (100 mg/kg) reduced histamine content from 83.8 ± 7.10 to 57.4 ± 3.58 µg/g and increased the rate of formation from 6.6 ± 1.00 to 17.7 ± 1.37 µg g$^{-1}$ 3 h$^{-1}$. During isoprenaline infusion, vagal stimulation induced only a slight increase in histamine formation to 9.0 ± 1.41 µg g$^{-1}$ 3 h$^{-1}$ and no effect was noted on histamine content. Propranolol reversed the inhibitory effect of isoprenaline on 2-deoxy-D-glucose-induced histamine mobilization (Table 1). It should be noted that isoprenaline alone increased the rate of histamine formation and slightly reduced histamine content (Table 1).

*Isoprenaline-induced alterations in serum gastrin and glucose*

Isoprenaline infusion (40 µg kg$^{-1}$ h$^{-1}$) induced a progressive increase in immunoreactive serum gastrin concentration from about 60 pg/ml in the fasting state to 130.8 ± 17.95 pg/ml after 2.5 h of infusion. Propranolol (2 mg/kg) administered at hourly intervals prevented the increase (Figure 5). The serum concentration of glucose in the fasting state was about 80 mg/100 ml and increased on isoprenaline infusion to a peak value of 115.1 ± 10.25 mg/100 ml after 60 minutes. On prolonged isoprenaline infusion, serum glucose concentration returned to control levels.

*The effect of isoprenaline on histamine-induced secretion*

The effect of isoprenaline (20 µg kg$^{-1}$ h$^{-1}$) on histamine-induced (1000 µg/h) gastric secretion was investigated in rats provided with Heidenhain pouches. This dose of isoprenaline was chosen since it effectively reduced the acid secretory response to pentagastrin (Lundell & Svensson, 1974). In six rats, isoprenaline given 30 min before and maintained during histamine infusion, inhibited acid secretion by about 40% (Figure 6a). In contrast, the stimulatory effect of histamine on pepsin secreting cells was slightly augmented by isoprenaline (Figure 6b).

In another group of six Heidenhain pouch rats, the effect of isoprenaline was studied on a plateau secretion induced by histamine. Histamine infusion failed to elicit a stable plateau secretion, instead the secretory response faded with time of infusion. Isoprenaline (20 µg kg$^{-1}$ h$^{-1}$) reduced the acid response, a reduction that reached statistical significance 1 and 1.5 h after the start of isoprenaline

**Table 1** Histamine formation (µg g$^{-1}$ 3 h$^{-1}$) and content (µg g$^{-1}$) 2 h after intravenous injection of 2-deoxy-D-glucose (2DG, 100 mg/kg) alone and in combination with isoprenaline (Iso, 40 µg kg$^{-1}$ h$^{-1}$) or isoprenaline plus propranolol (Prop, 2 mg/kg)

| | *Controls* | *2DG* | *2DG+Iso* | *2DG+Iso +Prop* | *Iso* |
|---|---|---|---|---|---|
| Histmine formation | 6.6 ± 1.00 | 17.7 ± 1.37 | 9.0 ± 1.41 | 17.3 ± 1.82 | 9.4 ± 2.90 |
| Histamine content | 83.8 ± 7.10 | 57.4 ± 3.58 | 90.0 ± 13.40 | 58.3 ± 7.03 | 75.2 ± 11.45 |

Isoprenaline infusion started 30 min before the injection of 2-deoxy-D-glucose. Propranolol was injected 10 min before the start of isoprenaline infusion and the injection was repeated at hourly intervals. The controls were given saline only. The mean and s.e. mean are calculated from determinations on six rats in each group.

P 25829

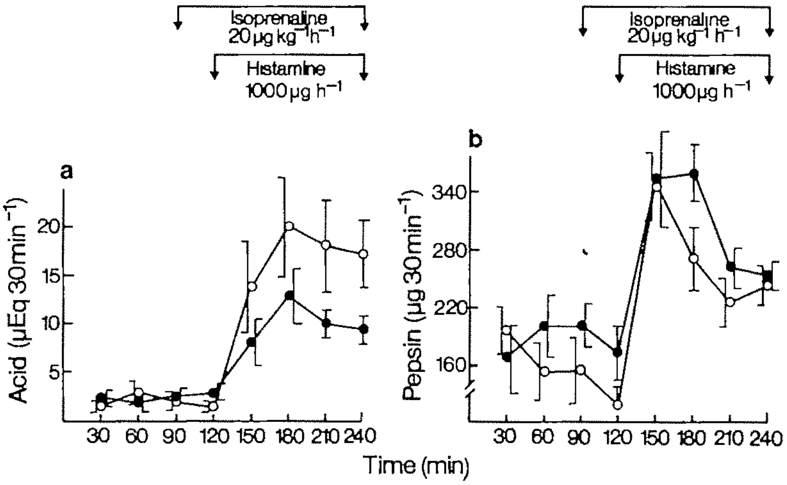


**Figure 6** (a) Acid and (b) pepsin secretion in response to histamine infusion (1000 μg $h^{-1}$) alone (○) or combined with isoprenaline (20 μg $kg^{-1}$ $h^{-1}$) (●). Each point represents the mean of one determination in each of six Heidenhain pouch rats. The vertical lines show s.e. mean.

infusion ($P < 0.05$) (Figure 7). Propranolol blocked the effect of isoprenaline (Figure 7), but the β-receptor antagonist did not influence the acid secretory response to histamine alone.

The stimulant effect of histamine on pepsin secretion was slight on prolonged infusion. Neither isoprenaline nor propranolol affected the pepsin secretory response.

### *The effect of isoprenaline on methacholine-induced secretion*

The secretory response to methacholine alone (1 μg/h) or in combination with isoprenaline (20 μg $kg^{-1}$ $h^{-1}$) was studied in six Heidenhain pouch rats. Methacholine induced an acid secretory response of about 15 μEq/30 min with a tendency to increase with time of infusion. Isoprenaline infusion did not significantly inhibit the secretory response (Figure 8a).

The potent stimulation of pepsin secretion by methacholine is illustrated in Figure 8b. Isoprenaline augmented this effect of methacholine, an increase that reached statistical significance ($P < 0.05$) during the last 90 min of isoprenaline administration.

## Discussion

Isoprenaline is known to inhibit histamine release from sensitized lung tissue (Assem & Schild, 1969) and leucocytes (Lichtenstein & Margolis, 1968). In the leucocytes, the β-receptor agonist also restrains the rate of histamine formation (Assem, 1974). Similar effects of isoprenaline have been noted on histamine release and formation in the rat gastric mucosa upon stimulation with pentagastrin (Lundell & Svensson, 1974). Since isoprenaline also inhibited the acid secretory response to pentagastrin, Lundell & Svensson (1974) tentatively suggested that isoprenaline inhibited acid secretion by restraining histamine mobilization. Curwain, Holton, McIsaac & Spencer (1974) reached a similar conclusion from their studies in the dog.

In dogs, cholinergically-induced acid secretion is extremely susceptible to inhibition by sympathomimetic amines (Harries, 1957; Pradhan &

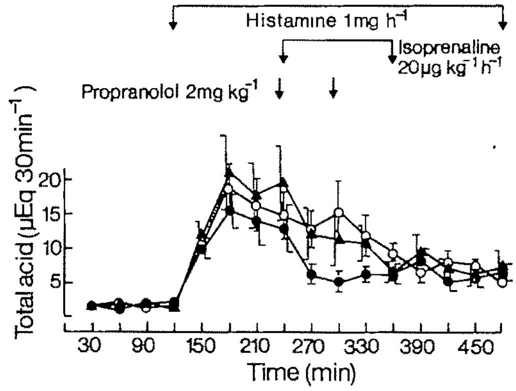


**Figure 7** Acid secretion in response to histamine (1 mg $h^{-1}$) alone (○) or combined either with isoprenaline (20 μg $kg^{-1}$ $h^{-1}$) (●) or isoprenaline plus propranolol (2 mg $kg^{-11}$) (▲). Propranolol was injected as indicated by the arrows. Each point represents the mean of one determination in each of six Heidenhain pouch rats. The vertical lines show s.e. mean.

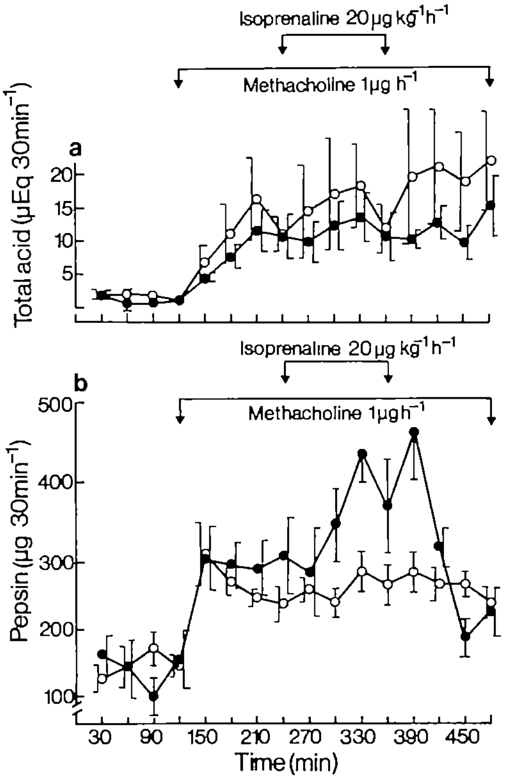


**Figure 8** (a) Acid and (b) pepsin secretion in response to methacholine (1 µg $h^{-1}$) alone (O) or combined with isoprenaline (20 µg $kg^{-1}$ $h^{-1}$) (●). Each point represents the mean of one determination in each of six Heidenhain pouch rats. The vertical lines show s.e. mean.

Wingate, 1962). In the present study, isoprenaline infusion almost abolished acid secretion in the Pavlov pouch induced by feeding and substantially reduced the response to 2-deoxy-D-glucose. These observations raise the question whether the mechanism behind the effect of the β-receptor agonist is a reduction in the mobilization of gastric mucosal histamine.

2-Deoxy-D-glucose injection is known to be followed by an increase in the vagal tone above that prevailing in the interdigestive state, allegedly by stimulation of nuclei in the hypothalamus (Colin-Jones & Himsworth, 1970). Central vagal excitation effected in this way elicited a reduction in mucosal histamine content and a substantial increase in the rate of formation of the amine. In the presence of the antrum, 100 mg/kg of 2-deoxy-D-glucose evokes an immediate increase in serum gastrin (Lundell & Nilsson, unpublished observations). In the absence of the antrum, vagal excitation does not alter the serum gastrin level but is still effective in increasing the rate of mucosal histamine formation (Rosengren & Svensson, 1969; Lundell, 1975a). Accordingly, enhanced gastrin secretion seems to be only partly responsible for the observed effects of vagal stimulation on mucosal histamine mobilization. From the present results it is obvious that isoprenaline almost completely prevented the combined effect of the vagus nerve and endogenous gastrin on the mobilization of mucosal histamine.

Interdigestive acid secretion is sustained by both antral gastrin release and vagus nerve activity. In the rat, the latter mechanism appears to be predominant (Svensson, 1970; Lundell, 1975b). Isoprenaline strongly inhibited interdigestive secretion. Surprisingly, after 2.5 h of isoprenaline infusion, mucosal histamine content was slightly decreased and the rate of formation increased. However, it should be pointed out that isoprenaline induced a progressive increase in serum gastrin concentration, thus providing a potent stimulus for histamine mobilization, which was apparently counterbalanced by the β-receptor agonist (see also Hayes, Ardill, Kennedy, Shanks & Buchanan, 1972; Stadil & Rehfeld, 1973). However, these results are irreconcilable with a primary site of secretory inhibition by isoprenaline on histamine mobilization. Furthermore, exogenous histamine failed to prevent the inhibitory effect of β-adrenoceptor stimulation on the 2-deoxy-D-glucose response.

In dogs, isoprenaline has little effect on histamine-induced secretion but inhibits pentagastrin-induced secretion and increases the ratio of gastric mucosal blood flow to acid secretion (Curwain & Holton, 1972). In the rat Heidenhain pouch, histamine-induced secretion was inhibited by isoprenaline infusion, although isoprenaline was more effective when secretion was stimulated by pentagastrin (Lundell & Svensson, 1974). Since no corresponding effect was noted on methacholine-induced secretion, isoprenaline may interact with the histamine $H_2$-receptor on the parietal cell. However, such interaction has not been demonstrated on other target organs having $H_2$-receptors (Black *et al.*, 1973; Pöch, Kukovetz & Scholz, 1973). Nevertheless, isoprenaline has been reported to inhibit histamine-induced acid secretion in the bull frog isolated mucosa (Thorpe, Frusco, Bass & Hug, 1971), indicating a direct effect on the parietal cell.

The secretory responses studied here in the Pavlov pouch are predominantly governed by vagus nerve activity. Another mechanism, besides those discussed above, contributing to the mode of inhibition of isoprenaline may be a central or peripheral depression of vagal activity. Although it has been reported that an artificial elevation of blood glucose concentration inhibits the secretory response to 2-deoxy-D-glucose (Hirschowitz & Sachs, 1965; Himsworth & Colin-Jones, 1969), it is unlikely that the rather small increase in serum glucose that we observed on isoprenaline infusion would diminish the secretory rate during the interdigestive state and upon feeding or 2-deoxy-D-glucose injection. Catecholamines and sympathetic nerve stimulation are known to inhibit cholinergically-induced excitatory motor responses in the gastric and intestinal wall. This effect seems to be at least partly attributable to an inhibited acetylcholine release from the cholinergic neurones (Jansson & Martinsson, 1966; Paton & Vizi, 1969; Kosterlitz, Lydon & Watt, 1970). The present finding that isoprenaline inhibited vagally- and histamine-induced secretory responses may be reconcilable with restrained acetylcholine release, since it has been shown that the secretory response to histamine is enhanced by cholinergic stimulation and is inhibited by atropine (Johansson, Lundell & Svensson, 1971; Johansson *et al.*, 1972). However, it should be recalled that catecholamines seem to prevent acetylcholine release from cholinergic neurones by an α-receptor mechanism (Paton & Vizi, 1969; Kosterlitz *et al.*, 1970).

In the Pavlov pouch, pepsin secretion is excited only by cholinergic stimulants (Svensson, 1970; Johansson *et al.*, 1972). The denervated peptic cells are responsive also to histamine and gastrin. In the rat Heidenhain pouch, isoprenaline enhanced the pepsin secretory response to all stimuli so far investigated (see also Lundell & Svensson, 1974). By contrast, in the innervated pouch pepsin secretion stimulated by 2-deoxy-D-glucose was inhibited by isoprenaline. These results contrast with those obtained in dogs provided with gastric fistulae (Grechishkin, 1970). It would thus appear that in the rat denervation profoundly alters the secretory behaviour of the peptic cells to β-adrenoceptor stimulation.

This work was supported by grants from the Swedish Medical Research Council, the Medical Faculty, University of Lund and Leo Ltd, Helsingborg Research Foundation.

## References

ALPHIN, R.S. & LIN, T.M. (1959). Preparation of chronic denervated gastric pouches in the rat. *Am. J. Physiol.*, **197**, 257–259.

ASSEM, E.S.K. (1974). Effect of sympathomimetic amines on the histamine forming capacity of human leucocytes. *Br. J. Pharmac.*, **52**, 213–218.

ASSEM, E.S.K. & SCHILD, H.O. (1969). Inhibition by sympathomimetic amines of histamine release induced by antigen in passively sensitized human lung. *Nature, Lond.*, **224**, 1028–1029.

BITSCH, V. (1966). A modification of the Hunt method for estimating peptic activity in gastric juice. *Scand. J. clin. Lab. Invest.*, **18**, 357–358.

BLACK, J.W., DUNCAN, W.A.M., EMMETT, J.C., GANELLIN, C.R., HESSELBO, T., PARSONS, M.E. & WYLLIE, J.H. (1973). Metiamide – an orally active histamine $H_2$-receptor antagonist. *Agents and Actions*, **3**, 133–137.

BRAY, G.A. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analyt. Biochem.*, **1**, 279–285.

COLIN-JONES, D.G. & HIMSWORTH, R.L. (1970). The location of the chemoreceptor controlling gastric acid secretion during hypoglycaemia. *J. Physiol., Lond.*, **206**, 397–409.

CURWAIN, B.P. & HOLTON, P. (1972). The effects of isoprenaline and noradrenaline on pentagastrin-stimulated gastric acid secretion and mucosal blood flow in the dog. *Br. J. Pharmac.*, **46**, 225–233.

CURWAIN, B.P., HOLTON, P., McISACC, R.L. & SPENCER, J. (1974). Can the actions of adrenoceptor agonists and antagonists on pentagastrin-induced gastric secretion be due to their effects on histamine formation? *Br. J. Pharmac.*, **51**, 217–223.

FELDBERG, W. & TELESNIK, J. (1953). Reduction of tissue histamine by compound 48/80. *J. Physiol., Lond.*, **120**, 550–568.

GRECHISHKIN, L.L. (1970). Catecholamines and 5-hydroxytryptamine effect on the gastric secretion. *Farmakologija i Toksikologija*, **30**, 612–615.

HAKANSON, R. & LIEDBERG, G. (1970). The role of endogenous gastrin in the activation of gastric histidine decarboxylase in the rat. Effect of antrectomy and vagal denervation. *Eur. J. Pharmac.*, **12**, 94–103.

HARRIES, E.H.L. (1957). The mode of action of sympathomimetic amines in inhibiting gastric secretion. *J. Physiol., Lond.*, **138**, 48–50P.

HAYES, J.R., ARDILL, J., KENNEDY, T.L., SHANKS, R.G. & BUCHANAN, K.D. (1972). Stimulation of gastrin release by catecholamines. *Lancet*, **i**, 819–821.

HENNINGSSON, S., LUNDELL, L. & ROSENGREN, E. (1974). Comparative study of two isotopic methods determining histamine formation *in vitro*. *Biochem. Pharmac.*, **23**, 2671–2678.

HIMSWORTH, R.L. & COLIN-JONES, D.G. (1969). Factors which determine the gastric secretory response to 2-deoxy-D-glucose. *Gut*, **10**, 1015–1019.

HIRSCHOWITZ, B.I. & SACHS, G. (1965). Vagal gastric secretory stimulation by 2-deoxy-D-glucose. *Am. J. Physiol.*, **209**, 452–460.

HUNT, J.N. (1948). A method for estimating peptic activity in gastric contents. *Biochem. J.*, **42**, 104–109.

JANSSON, G. & MARTINSSON, J. (1966). Studies on the ganglionic site of action of sympathetic outflow to the stomach. *Acta physiol. scand.*, **68**, 184–192.

JOHANSSON, I., LUNDELL, L., ROSENGREN, E. & SVENSSON, S.E. (1972). Gastric secretion and its facilitation as related to gastric mucosal histamine. *J. Physiol., Lond.*, **226**, 431–452.

JOHANSSON, I., LUNDELL, L. & SVENSSON, S.E. (1971). Inhibition of gastric secretion by atropine in conscious rats. *J. Physiol., Lond.*, **217**, 723–735.

KAHLSON, G., ROSENGREN, E., SVAHN, D. & THUNBERG, R. (1964). Mobilization and formation of histamine in the gastric mucosa as related to acid secretion. *J. Physiol., Lond.*, **174**, 400–416.

KOBAYASHI, Y. (1963). Determination of histidine decarboxylase activity by liquid scintillation counting of $^{14}CO_2$. *Analyt. Biochem.*, **5**, 284–290.

KOSTERLITZ, H.W., LYDON, R.J. & WATT, A.J. (1970). The effects of adrenaline, noradrenaline and isoprenaline on inhibitory $\alpha$- and $\beta$-adrenoceptors in the longitudinal muscle of the guinea-pig ileum. *Br. J. Pharmac.*, **39**, 398–413.

LICHTENSTEIN, L.M. & MARGOLIS, S. (1968). Histamine release *in vitro*: Inhibition by catecholamines and methylxanthines. *Science*, **161**, 902–903.

LUNDELL, L. (1973). Mobilization of gastric mucosal histamine and gastric secretion after $H_2$-receptor blockade. *Br. J. Pharmac.*, **49**, 715–717.

LUNDELL, L. (1974a). Histamine metabolism of the gastric mucosa following antrectomy. *J. Physiol., Lond.*, **241**, 437–451.

LUNDELL, L. (1974b). Acid secretion and mobilization of gastric mucosal histamine on combined cholinergic and pentagastrin stimulation. *Agents and Actions*, **4**, 211–216.

LUNDELL, L. (1975a). Elucidation by a $H_2$-receptor antagonist of the significance of mucosal histamine mobilization in exciting acid secretion. *J. Physiol., Lond.*, **244**, 365–383.

LUNDELL, L. (1975b). Alterations in secretory patterns following antrectomy in rats with Pavlov pouches. *J. Physiol., Lond.*, **245**, 737–754.

LUNDELL, L. & SVENSSON, S.E. (1974). Implication of gastric mucosal histamine in inhibition by isoprenaline of pentagastrin-induced gastric secretion. *Br. J. Pharmac.*, **52**, 69–75.

NILSSON, G. (1975). Increased plasma gastrin levels in connection with inhibition of gastric acid responses to sham feeding following bulbar perfusion with acid in dogs. *Scand. J. Gastroenterol.*, **10**, 273–277.

PATON, W.D.M. & VIZI, E.S. (1969). The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip. *Br. J. Pharmac.*, **35**, 10–28.

PÖCH, KUKOVETZ, W.R. & SCHOLZ, N. (1973). Specific inhibition by Burimamide of histamine effects on myocardial contraction and cyclic AMP. *Naunyn-Schmiedebergs Arch. Pharmak.*, **280**, 223–228.

PRADHAN, S.N. & WINGATE, H.N. (1962). Effects of adrenergic agents on gastric secretion in dogs. *Archs int. Pharmacodyn. Thér.*, **140**, 399–408.

REHFELD, J.F., STADIL, F. & VIKELSØE, J. (1974). Immunoreactive gastrin components in human serum. *Gut*, **15**, 102–111.

ROSENGREN, E. & SVENSSON, S.E. (1969). The role of the antrum and the vagus nerve in the formation of gastric mucosal histamine. *J. Physiol., Lond.*, **205**, 275–288.

STADIL, F. & REHFELD, J.F. (1973). Release of gastrin by epinephrine in man. *Gastroenterology*, **65**, 210–215.

SVENSSON, S.E. (1970). The secretory pattern of three stomach preparations in the rat. *J. Physiol., Lond.*, **207**, 329–350.

THORPE, C.D., FRUSCO, R.A., BASS, P. & HUG, Jr. C.C. (1971). Effects of catecholamines on acid secretion by bullfrog isolated gastric mucosa. *Surg. Forum*, **12**, 317–319.

*(Received July 8, 1975.*
*Revised April 4, 1976.)*

traperitoneal drug infusion. Control rats received repeated injections of 0.9% w/v NaCl solution (saline). The halothane sleeping time was first determined 4 h after the last drug injection and at intervals thereafter until three successive values indicated a return to normal values.

Because of the interesting nature of the changes in sensitivity of the CNS which were indicated by our results, the following additional measurements were made in some instances only: (1) brain halothane concentration on awakening; (2) sleeping time following i.c.v. injection of pentobarbitone, and (3) bemegride-induced seizure threshold. With pentobarbitone only, the rate at which the drug was excreted was determined by measurement of brain and liver pentobarbitone and pentobarbitone metabolite concentrations in groups of animals killed at intervals following repeated intraperitoneal injections of [$^{14}$C]-pentobarbitone administered in exactly the same way (90 mg kg$^{-1}$ 10 h$^{-1}$) as in the experiment described above.

### *Determination of brain halothane concentration*

Rats were killed by cervical dislocation on awakening after exposure to halothane and the brain rapidly removed (<1 min) and placed in a previously weighed screwcapped MSE homogenizer bottle (25 ml) containing 2 ml *n*-heptane. The container was reweighed and the brain weight obtained by difference. Further *n*-heptane was then added to give 2 ml g$^{-1}$ tissue and the brain was homogenized for 30 s at full speed with an MSE homogenizer. The homogenates were spun at 5,000 rev/min for 5 min in an MSE minor centrifuge and the superantant solution was then decanted and stored at −20°C in a stoppered tube until required for assay. On each occasion, standards were prepared by homogenization of brain taken from saline pretreated rats with known amounts of halothane in *n*-heptane (2 ml heptane g$^{-1}$ brain tissue). The final halothane concentrations of these standards was between 0.0184 mg ml$^{-1}$ and 0.1472 mg ml$^{-1}$. All assays were performed within 12 days of preparation of the homogenates, during which time there had been a mean loss of $17.6 \pm 2.0\%$ halothane ($n=5$) compared with freshly prepared homogenates. The halothane was assayed by gas chromatography using a PYE series 104 chromatograph with flame ionization detector. Samples (10 µl) were injected onto a 1.5 m column packed with Poropak Q and maintained at a temperature of 170°C. The carrier gas was argon at a flow rate of 60 ml min$^{-1}$; hydrogen and air flow rates were 60 ml min$^{-1}$ and 200 ml min$^{-1}$ respectively. Halothane concentrations were determined from a calibration curve prepared using the standard supernatant solutions.

### *Determination of brain and liver [$^{14}$C]-pentobarbitone concentration*

Animals were killed by stunning and subsequent cervical dislocation. The brain and liver were removed, weighed and each tissue was homogenized in 7 ml pH 5.0 phosphate buffer. [$^{14}$C]-pentobarbitone was extracted by shaking for 30 min with 30 ml petroleum ether (40–60) 98.5%/iso-amyl alcohol 1.5%. After centrifugation at 2,000 rev/min for 10 min (MSE major), 10 ml of the petroleum ether layer was removed for liquid scintillation counting. The remaining petroleum ether layer was aspirated and the aqueous layer was re-extracted with a further 30 ml of petroleum ether/iso-amyl alcohol. After re-centrifugation, the organic layer was aspirated, the surface of the lower (buffer) layer washed twice with 10 ml petroleum ether/iso-amyl alcohol and [$^{14}$C]-pentobarbitone metabolites were extracted from the remaining aqueous layer by shaking with 30 ml ethyl acetate for 30 minutes. After centrifugation, 10 ml of the ethyl acetate layer was removed for liquid scintillation counting. Standard amounts of [$^{14}$C]-pentobarbitone were taken through the same extraction procedure. Pentobarbitone metabolite concentrations were determined as the corresponding amounts of unchanged pentobarbitone.

### *Determination of sleeping time after i.c.v. injection of pentobarbitone*

Sleeping time determinations were performed on pentobarbitone and meprobamate-treated rats. Animals were lightly anaesthetized with ether, the surface of the skull was exposed and a small burr hole was made in the skull at a point 2.5 mm lateral and 1.0 mm posterior to the bregma. The rats were allowed to recover for 10 min and 800 µg sodium pentobarbitone in 30 µl water was injected into the left lateral ventricle. Injection was carried out with a 50 µl Hamilton syringe connected to a size 25 g × ⅝ needle fitted with a polythene tubing 'spacer' allowing injection at a depth of 4 mm below the surface of the skull. The times at which animals lost and regained their righting reflex were recorded with a stopwatch.

### *Determination of bemegride-induced seizure threshold*

The bemegride seizure threshold was determined in groups of pentobarbitone-treated rats. The CD$_{50}$ for bemegride was calculated by treating groups of 6 rats at 4 dose levels according to the method of Weil (1952). Animals failing to convulse within 5 min of intraperitoneal injection were scored as negative. The results were calculated as the mean with 95% confidence limits.

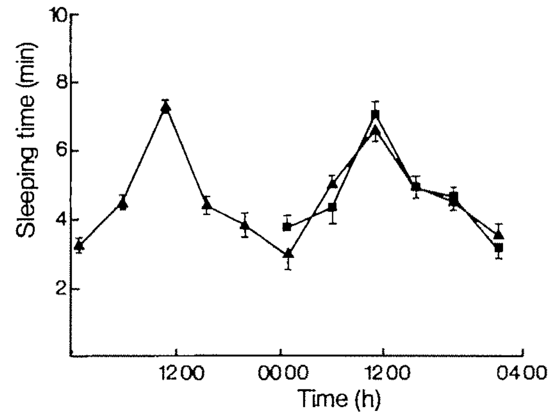


**Figure 1** Diurnal variation in, and effect of repeated exposure to halothane on, halothane-induced sleeping time. Each point represents the mean of 6 observations. Vertical lines show s.e. mean. (▲) Group 1, exposed repeatedly for 50 h; (■) Group 2, exposed only on day 2.

**Table 1** Diurnal variation in sensitivity to halothane

| *Time* | *Sleeping time* • *(min)* | *Brain halothane conc. on awakening* *(μg g⁻¹)* |
|---|---|---|
| 01 h 00 min | 2.50±0.14 (6) | 167± 7 (6) |
| 11 h 00 min | 4.45±0.10 (6) | 126±11 (6) |
| | $P<0.001$ | $P<0.01$ |

Figures represent mean±s.e. mean with number of observations in parentheses.

## Results

Under the conditions adopted, there was only a small intra- and inter-group variation in sleeping time on any one occasion. The end-point, i.e., awakening from the anaesthetic, was very sharp. Animals passed from the flacid immobile state to one of considerable locomotor activity within a few seconds. However a marked diurnal variation in sleeping time was observed (Figure 1). There was a considerable decrease in sensitivity to halothane during the night (dark phase) compared with during the day (light phase) and this diurnal variation in sleeping time was due, at least in part, to an altered sensitivity of the CNS to the anaesthetic. This was indicated by the observation that the brain halothane concentration found in rats awakening from the anaesthetic at 11 h 00 min was lower than the concentration found in animals awakening at 01 h 00 min (Table 1). Repeated sleeping time determinations on the same group of animals did not induce a tolerance, since the sleeping time on the eleventh exposure to halothane within a 48 h period was not significantly different from the sleeping time on the first occasion (Figure 1). Furthermore, the sensitivity of the second group of rats exposed only during the second 24 h period was the same as that of animals repeatedly exposed over the whole 48 h period (Figure 1). In a separate experiment, brain halothane concentrations found on awakening confirmed that repeated exposure to halothane did not affect the sensitivity of the CNS to the anaesthetic (Table 2). Thus, animals exposed on the twelfth occasion during a 50 h cycle awakened with the same brain halothane concentration as rats exposed only twice during the same period.

To determine whether pretreatment with small doses of CNS stimulant or depressant drugs would affect the sleeping time, rats were injected with amphetamine, pentobarbitone or nitrazepam (Table 3). Amphetamine pretreatment shortened and pentobarbitone and nitrazepam pretreatment lengthened the sleeping time (Table 3). With pentobarbitone only, the sleeping time was determined at

**Table 2** Effect of repeated halothane inhalation on halothane-induced sleeping time and brain halothane concentration on awakening

| | *Number of exposures to halothane* | *Time (h)* | *Sleeping time (min)* | *Brain halothane conc. on awakening (μg g⁻¹)* |
|---|---|---|---|---|
| Group 1 | 1 | 0 | 4.35±0.13 (6) | — |
| Group 2 | 1 | 0 | 4.20±0.15 (6) | — |
| Group 1 | 2 | 50 | 5.46±0.22 (6) | 120± 6 (6) |
| Group 2 | 12 | 50 | 5.81±0.20 (6) | 111±10 (6) |

Figures represent mean±s.e.mean with number of observations in parentheses.

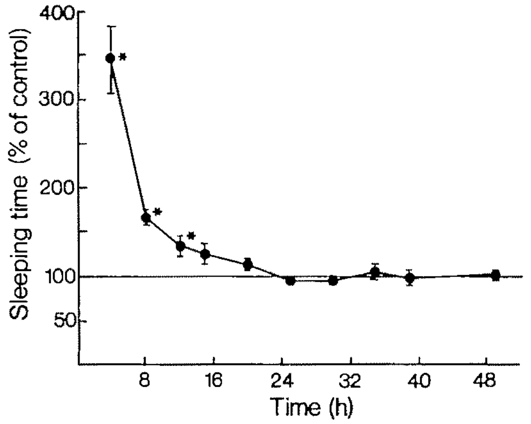


Figure 2 Halothane-induced sleeping time at intervals following a single intraperitoneal injection of pentobarbitone sodium (30 mg $kg^{-1}$). Each point represents the mean of 6 animals in comparison with 6 saline pretreated rats. Vertical lines show s.e. mean. The asterisks denote $P<0.05$ in comparison with control animals (Student's *t* test).

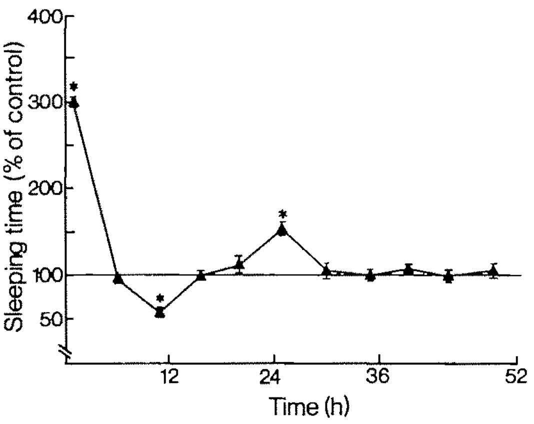


Figure 3 Halothane-induced sleeping time at intervals after repeated injections of amylobarbitone sodium (225 mg $kg^{-1}$ 10 $h^{-1}$). Each point represents the mean of 6 animals in comparison with 6 saline pretreated rats. Vertical lines show s.e. mean. Zero time on the horizontal axis is 4 h after the last injection of amylobarbitone. Asterisks denote $P<0.05$ in comparison with control animals (Student's *t* test).

intervals after recovery from a single intraperitoneal injection of an anaesthetic dose (30 mg/kg) in order to follow the time course of effect on halothane sleeping time (Figure 2). (In Figures 2, 3, and 4 the sleeping times are expressed as a percentage of control values, thus eliminating the diurnal variation and facilitating the comparison between drug pretreated and control animals. Each point on the graphs represents a comparison between drug and saline pretreated rats which had been exposed to halothane on the same occasion.) Our results show that the sleeping time was initially prolonged and that it gradually decreased to reach control values after 24 hours.

The sensitivity to halothane was not affected by the stimulation of hepatic drug-metabolizing enzyme activity produced by pretreatment of rats for four days with phenobarbitone or by the inhibition of enzyme activity produced by a single intraperitoneal injection of SKF 525A (Table 3).

The effect of repeated drug administration was determined on groups of rats which had been kept anaesthetized for approximately 12 h by repeated injection of amylobarbitone, pentobarbitone or meprobamate. The effect on halothane sleeping time was similar with all three drugs (Figures 3 and 4). The first sleeping time, determined 4 h after the last

**Table 3** Effect of drug pretreatment on halothane-induced sleeping time

| *Drug* | *Dose* | *Duration of pretreatment* | *Sleeping time (min)* |
|---|---|---|---|
| Control | | | 6.9 ± 0.2 (4) |
| Amphetamine sulphate | 1 mg $kg^{-1}$ | 10 min | 2.6 ± 0.2 (3)* |
| Pentobarbitone sodium | 5 mg $kg^{-1}$ | 5 min | 9.2 ± 0.1 (3)* |
| Nitrazepam | 1 mg $kg^{-1}$ | 15 min | 10.8 ± 1.2 (3)* |
| Control | | | 5.3 ± 0.1 (5) |
| Phenobarbitone sodium | 10 mg $kg^{-1}$ $day^{-1}$ | 4 days | 5.4 ± 0.2 (4) |
| S.K.F. 525A | 15 mg $kg^{-1}$ | 40 min | 5.7 ± 0.3 (4) |

Figures represent mean ± s.e. mean with number of observations in parentheses.
* $P<0.05$ compared with control values (Student's *t* test).

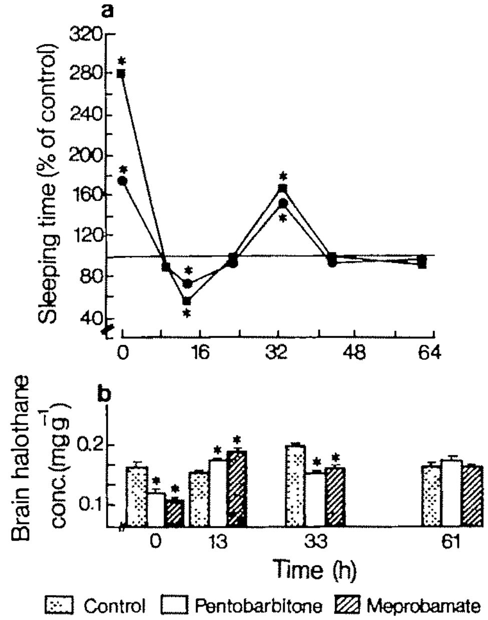


**Figure 4** (a) Halothane-induced sleeping time at intervals after repeated intraperitoneal injections of pentobarbitone sodium (●) (90 mg $kg^{-1}$ 10 $h^{-1}$) and meprobamate (■) (800 mg $kg^{-1}$ 10 $h^{-1}$). Each point represents the mean of 6 animals in comparison with 6 saline pretreated rats. Zero time on the horizontal axis is 4 h after the last injection of hypnotic. Asterisks denote $P < 0.05$ in comparison with control animals (Student's *t* test). (b) Brain halothane concentration found on awakening at various times after exposure to pentobarbitone or meprobamate. Each histogram represents the mean ± s.e. mean of 6 observations. Zero time on the horizontal axis is 4 h after the last injection of hypnotic. Asterisks denote $P < 0.05$ in comparison with control animals (Student's *t* test).

injection of depressant, was significantly prolonged compared with that in saline pretreated rats. However, the response then rapidly fell to below control values, this tolerance lasting approximately 8 h in the case of amylobarbitone (Figure 3) and 16 h in the cases of pentobarbitone and meprobamate (Figure 4a). With all three drugs the tolerance was followed by a hypersensitivity to halothane which was maximal approximately 28 h after the last injection of amylobarbitone (Figure 3) and 36 h after pentobarbitone or meprobamate (Figure 4a). Thereafter the sensitivity returned to normal. This pattern of changing sensitivity to halothane, a tolerance followed by a hypersensitivity, was in marked contrast to the gradual return to normal found after a single injection of pentobarbitone (Figure 2). In order to correlate the sleeping times with brain halothane concentrations on wakening, groups of rats were killed on awakening at various times after pretreatment with either pentobarbitone or meprobamate. The brain halothane concentrations on awakening are shown in Figure 4b. Initially, when the sleeping time was prolonged, pretreated animals awakened with significantly lower brain halothane concentrations. At the times of halothane tolerance

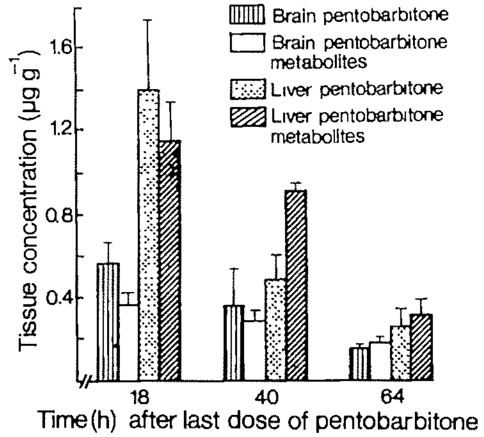


**Figure 5** Brain and liver pentobarbitone and pentobarbitone metabolite concentrations following repeated intraperitoneal injections of [$^{14}$C]-pentobarbitone sodium (90 mg $kg^{-1}$ 10 $h^{-1}$). Each histogram represents the mean ± s.e. mean of at least 4 observations.

**Table 4** Sleeping time to i.c.v. administered pentobarbitone in pentobarbitone and meprobamate-treated rats at times of tolerance and hypersensitivity to halothane

| | *Control* | *Pentobarbitone (90 mg $kg^{-1}$ 10 $h^{-1}$)* | *Meprobamate (800 mg $kg^{-1}$ 10 $h^{-1}$)* |
|---|---|---|---|
| At time of halothane tolerance | 5.5 ± 0.3 (6) | 3.2 ± 0.6 (4)<br>$P < 0.02$ | 3.2 ± 0.5 (6)<br>$P < 0.01$ |
| At time of halothane hypersensitivity | 4.6 ± 0.5 (8) | 6.8 ± 0.5 (8)<br>$P < 0.01$ | 6.2 ± 0.1 (8)<br>$P < 0.01$ |

Figures represent mean ± s.e. mean with number of observations in parentheses.

and hypersensitivity, the brain halothane concentrations found on awakening were significantly higher and lower respectively compared with saline pretreated rats. There was no difference between the brain halothane concentrations on awakening when sleeping times had returned to normal. With pentobarbitone only, brain and liver pentobarbitone and pentobarbitone metabolite concentrations were determined at intervals after repeated intraperitoneal injection of [$^{14}$C]-pentobarbitone (90 mg kg$^{-1}$ 10 h$^{-1}$). Brain pentobarbitone levels at the time of halothane tolerance (18 h after last dose of pentobarbitone) and hypersensitivity (40 h after last dose of pentobarbitone) were 0.55 μg g$^{-1}$ and 0.35 μg g$^{-1}$ respectively (Figure 5). The liver pentobarbitone concentrations were higher than those found in brain but the overall low levels of unchanged drug and metabolites (Figure 5) indicated that most of the drug had been eliminated even by the time of the first measurements.

The same pattern of results as was indicated by the halothane sleeping time determinations, an increase followed by a decrease in CNS excitability, was also found when the sleeping times following i.c.v. administered pentobarbitone and sensitivity to bemegride-induced seizures were considered. At the time of halothane tolerance, pentobarbitone and meprobamate pretreated animals were also tolerant to i.c.v. administered pentobarbitone (Table 4) and pentobarbitone-treated rats tended ($0.1 < P < 0.2$) to be more sensitive to intraperitoneally administered bemegride (control 15.2 (13.6–16.9); treated 12.2 (9.1–16.5) mean mg kg$^{-1}$ (95% confidence limits)). In contrast, a decreased excitability of the CNS was indicated at the time of halothane hypersensitivity since pentobarbitone and meprobamate pretreated rats were more sensitive to i.c.v. administered pentobarbitone (Table 4) and the threshold for bemegride-induced seizures tended to be higher ($0.05 < P < 0.1$) in pentobarbitone-treated animals (control 13.8 (12.2–15.6); treated 16.3 (14.3–18.5) mean mg kg$^{-1}$ (95% confidence limits)).

## Discussion

Our objectives in this study were as follows: firstly, to determine whether halothane-induced sleeping time could be used as a valid index of the sensitivity of the CNS to a depressant drug; secondly, to determine whether repeated sleeping time estimations on the same group of animals could be reliably used to follow the changes in sensitivity to halothane occurring with time, and thirdly, to ascertain whether a cross-tolerance to halothane would be induced by pretreatment of animals with other CNS depressant drugs, and if so, to determine the time course of such changes.

We have found that the halothane-induced sleeping time shows only a small inter- and intra-group variation when determined at any particular time but that there is a pronounced diurnal variation in sensitivity to the anaesthetic. A circadian variation in anaesthetic effectiveness is well documented (Moore Ede, 1973), and in the present experiments we have demonstrated that this is due, at least in part, to a variation in the sensitivity of the CNS to halothane. The diurnal variation in hepatic drug-metabolizing enzyme activity (Radzialowski & Bousquet, 1968), which is largely responsible for the variation in sensitivity to non-gaseous anaesthetics and hypnotic agents, does not appear to be involved in the change in sensitivity to halothane since we have found that inhibition or induction of drug-metabolizing enzyme activity does not affect the halothane-induced sleeping time. However, changes in absorption, distribution and excretion of the anaesthetic could also be involved.

Repeated determination of the sleeping time on the same group of rats over a period of 48 h did not induce a tolerance to halothane. This was indicated by the finding that after repeated exposure to anaesthetic the sleeping time and brain halothane concentration on awakening were the same as those found in control rats. Nevertheless, in all further experiments, control rats which had been exposed to halothane on each and every occasion were always included.

Thus, having established that the sleeping time was directly related to the sensitivity of the CNS to the anaesthetic, and was unaffected by alteration of drug-metabolizing capacity or by prior repeated exposure to the anaesthetic, we considered that such sleeping time determinations could be reliably used to study the changes in CNS sensitivity produced by various drug pretreatment regimes.

Anaesthetization of rats for approximately 12 h by repeated intraperitoneal injections of pentobarbitone, amylobarbitone or meprobamate induced a cross-tolerance to halothane and with all three drugs the tolerance was followed by a rebound hypersensitivity to the anaesthetic. That the halothane tolerance represented a true cellular tolerance induced by the prior exposure to the hypnotic drugs was indicated by the fact that such animals awakened with higher brain halothane concentrations, were also tolerant to i.c.v. administered pentobarbitone and, in the case of pentobarbitone, tended to be more sensitive to bemegride. Thus the 12 h period of anaesthesia appeared to have induced a withdrawal hyperexcitability of the CNS. No such hyperexcitability was produced by the 2 h period of anaesthesia induced by the single injection of pentobarbitone.

With the same methodology, the period of halothane hypersensitivity was found to coincide with a period of decreased CNS excitability since such animals were more sensitive to i.c.v. administered pentobarbitone and, with pentobarbitone pretreatment, were less sensitive to bemegride. The possibility that the post-tolerance hypersensitivity could have been due to an additive effect of halothane and residual hypnotic drug was shown to be extremely unlikely, since it was found in a separate experiment that the brain pentobarbitone concentration at the time of hypersensitivity was only 0.55 $\mu g\ g^{-1}$. As animals normally awaken from pentobarbitone anaesthesia with brain concentrations of approximately 20 $\mu g\ g^{-1}$ (Stevenson & Turnbull, 1970) it would appear unlikely that the additional 0.55 $\mu g\ g^{-1}$ could cause a significant prolongation of halothane sleeping time.

We have also considered the possibility that the observed changes in sleeping time could have been due to a shift in the circadian rhythm of halothane sensitivity. However, the following (unpublished) findings suggest that this is not the case. First, we have found the same pattern of change in sleeping time, a tolerance followed by a hypersensitivity, regardless of whether the drug pretreatment is given during the light or dark phase; secondly, although at first disrupted by drug pretreatment, the circadian rhythm of locomotor activity returns to normal well before the return of normal halothane sensitivity; thirdly, not all hypnotics and tranquillizers induce a tolerance to halothane when administered in doses which cause a loss of righting reflex for the same time as used in the present experiments; and finally, quantitative differences in the magnitude of the tolerance can be obtained by pretreatment with various doses of pentobarbitone or by pretreatment on successive days.

As far as we are aware, this is the first report of a post-tolerance hypersensitivity of this type. Although Aston and co-workers have reported a hypersensitivity to barbiturate (Aston, 1966; 1973), the hypersensitivity in their experiments occurred at a much later stage after barbiturate administration. We have also reported on a barbiturate hypersensitivity which occurred some 3 weeks after stopping barbitone administration (Stevenson & Turnbull, 1968) but showed that a decreased drug-metabolizing capacity was the most likely explanation in that particular instance. In the present experiments our results clearly indicate that the prolonged halothane sleeping time is due to an increased sensitivity of the CNS to depressant drugs. The two most likely reasons why such a hypersensitivity has not been previously found are that the sensitivity of the CNS has been too infrequently assessed and also, that in most tolerance studies the methods used to measure changes in drug sensitivity are so affected by alteration of drug-metabolizing capacity that the more subtle changes occurring in the CNS tend to be masked.

It remains to be seen whether the induction of cross-tolerance to halothane by acute pretreatment with large doses of an hypnotic is indicative that such a drug would cause CNS changes, e.g. those responsible for the production of dependence, if it were to be given chronically and at a lower dose level. This possibility is currently being investigated. It is more than likely that the mechanisms responsible for the production of the cross-tolerance to halothane seen in the present acute experiments are the same as those which are responsible for the withdrawal phenomena seen in chronically pretreated animals. If this were to be the case, then the methodology that we have described above could be used to screen compounds for their dependence liability.

We are grateful to the Medical Research Council for financial assistance. We are also indebted to Dr D.T. Greenwood for much useful discussion.

## References

ASTON, R. (1966). Latent hypersensitivity to pentobarbital in the rat. *Proc. Soc. exp. Biol. Med.*, **121**, 623–626.

ASTON, R. (1973). Mechanisms contributing to barbiturate intolerance in rats. *Br. J. Pharmac.*, **49**, 527–533.

HO, I.K., YAMAMOTO, I. & LOH, H.H. (1975). A model for the rapid development of dispositional and functional tolerance to barbiturates. *Eur. J. Pharmac.*, **30**, 164–171.

KALANT, H., LeBLANC, A.E. & GIBBINS, R.J. (1971). Tolerance to, and dependence on, some non-opiate psychotropic drugs. *Pharmac. Rev.*, **23**, 135–191.

KATO, R. (1967). Analysis and differentiation of the mechanism in development of drug tolerance. *Jap. J. Pharmac.*, **17**, 499–508.

MOORE EDE, M.C. (1973). Circadian rhythms of drug effectiveness and toxicity. *Clin. Pharmac. Ther.*, **14**, 925–935.

RADZIALOWSKI, F.M. & BOUSQUET, W.F. (1968). Daily rhythmic variation in hepatic drug metabolism in the rat and mouse. *J. Pharmac. exp. Ther.*, **163**, 229–238.

STEVENSON, I.H. & TURNBULL, M.J. (1968). Hepatic drug-metabolizing enzyme activity and duration of hexobarbitone anaesthesia in barbitone-dependent and withdrawn rats. *Biochem. Pharmac.*, **17**, 2297–2305.

STEVENSON, I.H. & TURNBULL, M.J. (1970). The sensitivity of the brain to barbiturate during chronic administration and withdrawal of barbitone sodium in the rat. *Br. J. Pharmac.*, **39**, 325–333.

STEVENSON, I.H. & TURNBULL, M.J. (1974). A study of the factors affecting the sleeping time following intracerebroventricular administration of pentobarbitone sodium: effect of prior administration of centrally active drugs. *Br. J. Pharmac.*, **50**, 499–511.

TURNBULL, M.J. & WATKINS, J.W. (1975a). Further observations on the change in sensitivity to halothane induced by acute administration of central nervous system depressant drugs in the rat. *Br. J. Pharmac.*, **55**, 266–267P.

TURNBULL, M.J. & WATKINS, J.W. (1975b). The use of halothane-induced sleeping time as an index of central nervous system excitability. *Br. J. Pharmac.*, **55**, 307–308P.

WAHLSTROM, G. (1968). Hexobarbital (Enhexymalum NFN) sleeping times and E.E.G. threshold doses as measurements of tolerance to barbiturates in the rat. *Acta. pharmac. tox.*, **26**, 64–80.

WEIL, C.S. (1952). Tables for convenient calculation of median effective dose ($LD_{50}$ or $ED_{50}$) and instruction in their use. *Biometrics*, **8**, 249–255.

*(Received August 22, 1975.*
*Revised December 5, 1975.)*

*Br. J. Pharmac.* (1976), **58**, 37–41

# ADRENOCEPTORS OF THE GUINEA-PIG URINARY BLADDER

K.C. DAVE & A.S. DHATTIWALA
Department of Pharmacology, N.H.L. Municipal Medical College, Ellis Bridge, Ahmedabad 380 006, India

1 Adrenaline, noradrenaline and isoprenaline (5 μg/ml) did not affect the resting tone of the isolated urinary bladder of the guinea-pig.

2 The catecholamines (1–2 μg/ml) inhibited neuronally evoked contractions at various stimulation frequencies; the inhibition was maximum at 2 Hz and minimum at 50 Hz. Isoprenaline produced maximum inhibition.

3 Propranolol (0.5 μg/ml) completely blocked the catecholamine-induced inhibition at all the frequencies employed. The concentration-response curves of isoprenaline at 2, 10 and 50 Hz were characteristically shifted by propranolol (50 ng/ml). Phenoxybenzamine (0.2 μg/ml) was totally ineffective.

4 In some experiments adrenaline significantly raised the tone of the bladder exposed to propranolol; this effect could be blocked by phenoxybenzamine.

5 Acetylcholine-induced bladder contractions were inhibited by adrenaline (2 μg/ml); the inhibition was completely blocked by propranolol (0.5 μg/ml).

6 The results indicate the presence of an inhibitory $\beta$-adrenoceptor and suggest the possibility of an excitatory $\alpha$-adrenoceptor in guinea-pig urinary bladder.

## Introduction

Edvardsen & Setekleiv (1968) failed to observe any response of isolated urinary bladder strips of guinea-pig to exogenous catecholamines, suggesting the lack of adrenoceptors in the guinea-pig bladder. However, using the same preparation De Sy (1970) showed that while the resting tone remained unaffected, the exogenous catecholamines inhibited the contractions evoked by supraluminal electrical stimulation, and the results with adrenoceptor blocking agents indicated the presence of inhibitory $\alpha$- and $\beta$-adrenoceptors.

To our knowledge no further work has been done on the adrenoceptors in the guinea-pig bladder. The object of the present study was to characterize these adrenoceptors by observing the effect of catecholamines on the neuronally evoked bladder contractions *in vitro*. The isolated innervated urinary bladder preparation of the guinea-pig (Weetman, 1972) was found to be suitable for this purpose.

## Methods

The nerve-bladder preparations obtained from adult guinea-pigs of either sex were set up as described by Weetman (1972) in a 30 ml organ bath containing Tyrode solution maintained at 35 ± 1°C and bubbled with 5% $CO_2$ in $O_2$. The Tyrode solution had the following composition (g/l): NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, $NaH_2PO_4$ 0.05, $NaHCO_3$ 1.0 and glucose 1.0.

Contractions were recorded on a moving kymograph with isotonic frontal writing lever (load 1–1.5 g; magnification ×7). The bladders were neuronally stimulated with rectangular pulses (0.5 ms, 15–20 V) at different Hz for a variable duration so as to deliver 50–51 shocks every 2 minutes. Catecholamines have been reported to exhibit a frequency-dependent prejunctional inhibitory effect on cholinergic transmission (Vizi, 1968; Beani, Bianchi & Crema, 1969; Christ & Nishi, 1971; Knoll & Vizi, 1971). We, therefore, employed different frequencies (2 Hz–50 Hz) to distinguish a frequency-dependent effect, if any, of catecholamines on the guinea-pig bladder.

Each preparation served as its own control. The preparation was allowed to contract to neuronal stimulation for at least 20 min before the addition of any drug; the height of contraction was measured and was taken as the control. The contact period with the adrenoceptor blocking agent was 45 min and except for cumulative doses of isoprenaline, the tissue-catecholamine contact period did not exceed 4 minutes.

In a few experiments the effect of adrenaline on the

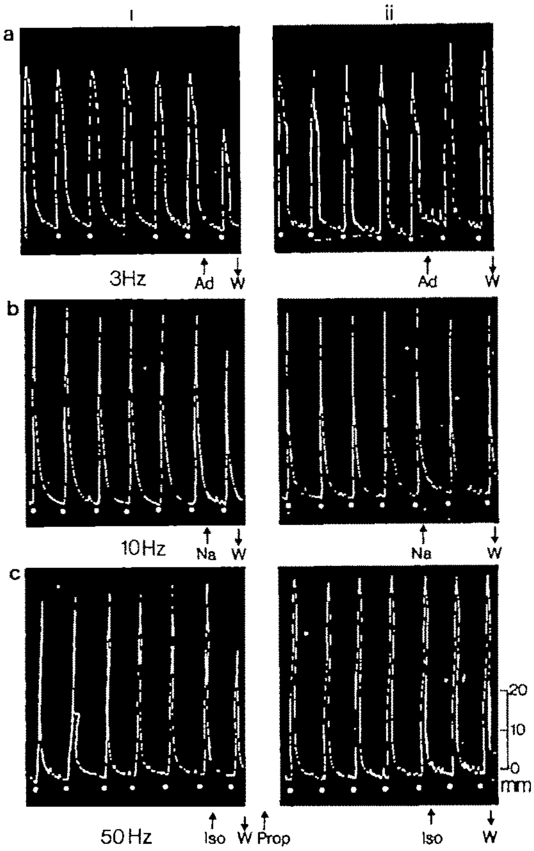


**Figure 1** Isolated urinary bladder of guinea-pig; neuronal stimulation at dots (rectangular pulses, 15 V, 0.5 ms, 50–51 shocks every 2 min at frequencies indicated below). Effect of adrenaline (2 µg/ml in (a)), noradrenaline (2 µg/ml in (b)) and isoprenaline (1 µg/ml in (c)) added at Ad, Na and Iso respectively. Propranolol (0.5 µg/ml in (a) and (c), 0.2 µg/ml in (b)) added at Prop. Interval between panels (i) and (ii), 40 min; W=wash. Note the raised tone by adrenaline in (a) in presence of propranolol. Figure retouched for printing.

acetylcholine-induced contraction was recorded and then repeated in the presence of propranolol.

### *Drugs*

(−)-Adrenaline acid tartrate (Sigma Chemicals, U.S.A.), (−)-noradrenaline acid tartrate (Fluka AG), isoprenaline sulphate (Burroughs Wellcome), propranolol hydrochloride (Inderal, ICI), phenoxybenzamine hydrochloride (SK & F) and acetylcholine bromide (Koch-Light Lab) were used and the concentrations refer to the salts.

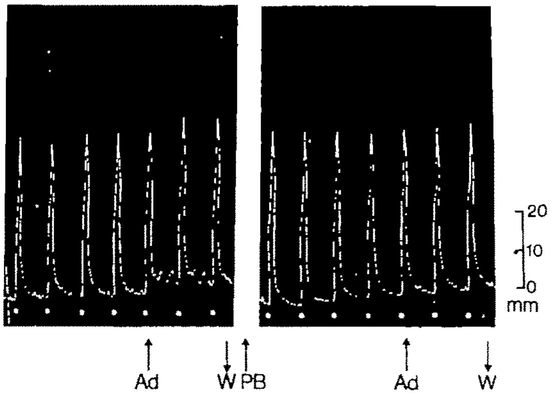


**Figure 2** Isolated urinary bladder of guinea-pig in Tyrode solution containing propranolol (0.5 µg/ml). Effect of phenoxybenzamine (PB, 0.2 µg/ml) on adrenaline (Ad, 2 µg/ml)-induced rise in tone in the neuronally stimulated bladder (stimulation at dots, rectangular pulses, 15 V, 0.5 ms, 10 Hz for 50 shocks/2 minutes). Interval between the panels, 40 minutes. W=wash. Figure retouched for printing.

## Results

Unless mentioned otherwise the number of experiments indicates experiments on separate bladders.

### *Effects of catecholamines on bladder tone and spontaneous rhythmic activity*

The resting tone of the preparation was unaltered by adrenaline, noradrenaline or isoprenaline in concentrations up to 5 µg/ml (10 tests, 5 bladders). Following exposure to propranolol (0.2–0.5 µg/ml) 6 out of 15 bladders responsed to adrenaline (2 µg/ml) with a contraction averaging 3.8 ± 0.21 mm (mean ± s.e.) e.g. panel a(ii) of Figure 1). In these 6 preparations the mean contraction to adrenaline in the absence of propranolol was 0.8 ± 0.12 mm, the difference being statistically significant ($P < 0.001$, Student's *t*-test for paired data). Phenoxybenzamine (0.2 µg/ml) blocked this effect (Figure 2). In only one out of 10 experiments noradrenaline (2 µg/ml) raised the tone by 2.5 mm in the presence of propranolol. In 3 out of 6 experiments isoprenaline (1 µg/ml) reduced the tone slightly in the presence of phenoxybenzamine (0.2 µg/ml) but this was not statistically significant ($0.05 < P < 0.1$).

Spontaneous rhythmic activity, when present, was markedly reduced after isoprenaline although the tone remained unaffected. In a few experiments the spontaneous activity was increased after adrenaline in the presence of propranolol.

**Table 1** Effects of catecholamines with and without propranolol (0.2–0.5 μg/ml) on the neuronally evoked contraction of the guinea-pig isolated urinary bladder (rectangular pulses, 15–20 V, 0.5 ms, 50–51 shocks/2 minutes)

| | | *Height of contraction (mm)* | | | *% Inhibition by catecholamine* | |
|---|---|---|---|---|---|---|
| *Catecholamine* | *Frequency* | *Control* | *Catecholamine without propranolol* | *Catecholamine with propranolol (P > 0.1)* | *Without propranolol* | *With propranolol* |
| Adrenaline (2 μg/ml) | 2 Hz | 19 ± 2 (9) | 10 ± 1 (9)‡ | 17 ± 2 (7) | 47 | 11 |
| | 3 Hz | 39 ± 3 (11) | 26 ± 2 (11)† | 35 ± 4 (8) | 33 | 10 |
| | 10 Hz | 49 ± 2 (27) | 36 ± 2 (27)‡ | 47 ± 2 (21) | 28 | 4 |
| | 50 Hz | 51 ± 2 (15) | 40 ± 1 (15)‡ | 50 ± 2 (12) | 22 | 2 |
| Noradrenaline (2 μg/ml) | 2 Hz | 20 ± 2 (10) | 11 ± 2 (10)† | 18 ± 3 (7) | 45 | 10 |
| | 3 Hz | 42 ± 4 (8) | 29 ± 3 (8)* | 44 ± 3 (6) | 30 | 0 |
| | 10 Hz | 50 ± 3 (9) | 40 ± 3 (9)* | 48 ± 3 (8) | 20 | 4 |
| | 50 Hz | 46 ± 3 (9) | 38 ± 2 (9)* | 46 ± 3 (7) | 17 | 0 |
| Isoprenaline (1 μg/ml) | 2 Hz | 17 ± 2 (8) | 7 ± 1 (8)‡ | 17 ± 2 (7) | 59 | 0 |
| | 10 Hz | 44 ± 2 (14) | 26 ± 2 (14)‡ | 40 ± 2 (13) | 41 | 9 |
| | 50 Hz | 49 ± 3 (15) | 36 ± 2 (15)† | 50 ± 4 (13) | 27 | 0 |

Numbers in parentheses indicate number of tests on minimum 5 bladders.

Significance between control and catecholamine with and without propranolol was calculated using Student's *t* test: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

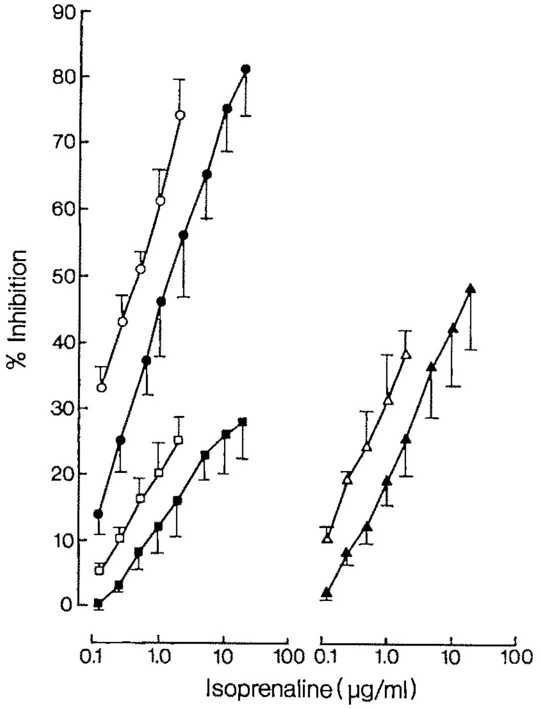


**Figure 3** Isolated urinary bladder of guinea-pig. Cumulative concentration effect curves for the isoprenaline-induced inhibition of neuronally evoked contractions: 2 Hz, (○); 50 Hz (□); 10 Hz (△); rectangular pulses, 10–20 V, 0.5 ms; total shocks, 50; every 2 minutes. Isoprenaline effect is expressed as the percentage inhibition of the control responses. Propranolol (50 ng/ml) was incubated with the tissue for 45 min before repeating the inhibitory effect of isoprenaline at 2 Hz (●), 50 Hz (■), and 10 Hz (▲). Vertical lines show s.e. mean of six experiments.

### *Effects of catecholamines with and without adrenoceptor blocking agents (Table 1)*

Adrenaline (2 μg/ml), noradrenaline (2 μg/ml) and isoprenaline (1 μg/ml) inhibited the neuronally evoked contractions in all the experiments and at all the stimulation frequencies. The inhibition was maximum at 2 Hz and minimum at 50 Hz. Maximum inhibition was produced by isoprenaline; adrenaline had a slightly greater inhibitory effect than noradrenaline.

Propranolol (0.2–0.5 μg/ml) and phenoxybenzamine (0.2 μg/ml) did not significantly affect the neuronally evoked contractions of the bladder. Irrespective of the frequency of stimulation propranolol (0.2–0.5 μg/ml) consistently and markedly blocked the adrenaline, noradrenaline and isoprenaline-induced inhibition (Figure 1). The blocking effect of propranolol persisted for at least 30 min after repeated washes.

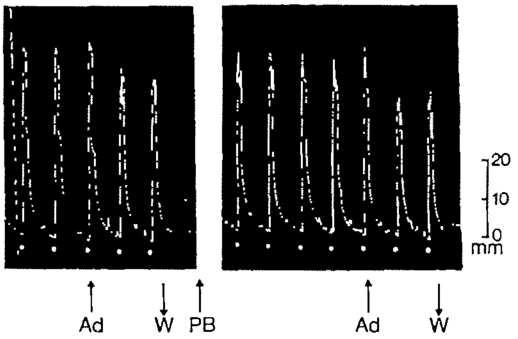


**Figure 4** Isolated urinary bladder of guinea-pig. Effect of phenoxybenzamine (PB, 0.2 μg/ml) on the adrenaline (Ad, 2 μg/ml)-induced inhibition of the neuronally stimulated bladder (stimulation at dots, rectangular pulses, 15 V, 0.5 ms, 5 Hz for 50 shocks/2 minutes). Interval between the panels, 40 min, W=wash. Figure retouched for printing.

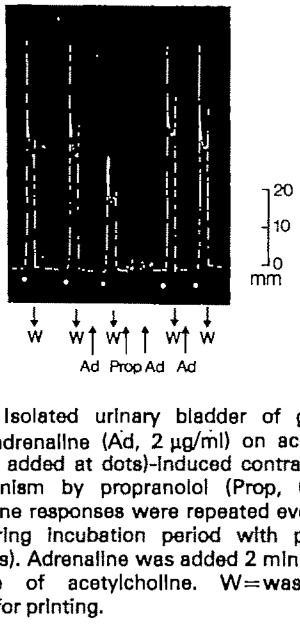


**Figure 5** Isolated urinary bladder of guinea-pig. Effect of adrenaline (Ad, 2 μg/ml) on acetylcholine (1.5 μg/ml, added at dots)-induced contractions and its antagonism by propranolol (Prop, 0.5 μg/ml). Acetylcholine responses were repeated every 15 min except during incubation period with propranolol (45 minutes). Adrenaline was added 2 min before the next dose of acetylcholine. W=wash. Figure retouched for printing.

In 6 experiments cumulative dose-response curves were obtained for isoprenaline alone and in the presence of propranolol (50 ng/ml), at 2, 10 and 50 Hz frequencies. Propranolol antagonized the isoprenaline-induced inhibition at all the three frequencies (Figure 3).

Phenoxybenzamine (0.2 μg/ml) did not antagonize the adrenaline or noradrenaline-induced inhibition in any of the experiments (10 tests, 5 bladders); in 4 tests (2 bladders) the contractions were reduced further (Figure 4). The blockade produced by propranolol could not be increased further by phenoxybenzamine.

### *Effect of adrenaline on acetylcholine-induced contractions*

Figure 5 shows the effect of adrenaline and its antagonism by propranolol on the acetylcholine-induced contraction of the guinea-pig bladder. In 5 tests on 3 bladders adrenaline (2 µg/ml) reduced the contraction produced by acetylcholine (1.5 µg/ml) to 55% (±3% s.e.) of the control; propranolol (0.5 µg/ml) antagonized this effect completely (the contraction reduced to 99% ± 1% s.e. of the control).

## Discussion

The isolated urinary bladder of the guinea-pig is a ganglion-containing cholinergically innervated preparation (Weetman, 1972; Weetman & Turner, 1973). Adrenaline and noradrenaline have been shown to inhibit the release of acetylcholine through their prejunctional action on the $\alpha$-adrenoceptor in the superior cervical ganglion of the rabbit (Christ & Nishi, 1971), in the guinea-pig ileum (Vizi, 1968; Knoll & Vizi, 1971) and the guinea-pig colon (Beani *et al.*, 1969): the inhibitory effect was evident at lower rather than at higher frequencies and could be blocked by $\alpha$-adrenoceptor blocking agents. The prejunctional inhibition by noradrenaline varies inversely with the frequency and the total number of shocks delivered during stimulation; the fewer the shocks and the lower the frequency applied, the greater is the inhibition (Knoll & Vizi, 1971). The number of shocks (50–51/2 min) was kept constant in our experiments so that though the maximum inhibition by the catecholamines was produced at the low frequency (2 Hz), propranolol could block the inhibitory effect irrespective of the frequency of stimulation whereas phenoxybenzamine was totally ineffective. Though isoprenaline lacks the prejunctional inhibitory effect (Vizi, 1968) it was maximally inhibitory in the present experiments. These results plus the characteristic shift of the dose-response curves of isoprenaline by propranolol indicate that the inhibitory effect of adrenaline, noradrenaline and isoprenaline observed on the neuronally stimulated guinea-pig urinary bladder *in vitro* is produced by the action of the catecholamines on inhibitory $\beta$-adrenoceptors. Inhibition of the acetylcholine-induced contraction of the isolated bladder by adrenaline and its antagonism by propranolol provide evidence for the post-junctional site of this inhibitory effect of the catecholamines.

A slight but significant rise in the tone induced by adrenaline in the presence of propranolol was observed in a few experiments, and its blockade by phenoxybenzamine may suggest the existence of excitatory $\alpha$-adrenoceptors in the guinea-pig bladder.

Gifts of phenoxybenzamine hydrochloride by SK & F and isoprenaline sulphate by Burroughs Wellcome & Co. are gratefully acknowledged. We thank Dr S.C. Pandya, Dean of N.H.L. Municipal Medical College, Ahmedabad, for providing the facilities to carry out the work.

## References

BEANI, L., BIANCHI, C. & CREMA, A. (1969). The effect of catecholamines and sympathetic stimulation on the release of acetylcholine from the guinea-pig colon. *Br. J. Pharmac.*, **36**, 1–17.

CHRIST, D.D. & NISHI, S. (1971). Site of adrenaline blockade in the superior cervical ganglion of the rabbit. *J. Physiol. Lond.*, **213**, 107–117.

DE SY, W. (1970). Receptor responses in isolated smooth muscle of the urinary bladder. *Arch. int. Pharmacodyn.*, **186**, 188–189.

EDVARDSEN, P. & SETEKLEIV, J. (1968). Distribution of adrenergic receptors in the urinary bladder of cats, rabbits and guinea-pigs. *Acta pharmac. et toxicol.*, **26**, 437–445.

KNOLL, J. & VIZI, E.S. (1971). Effect of frequency of stimulation on the inhibition by noradrenaline of the acetylcholine output from parasympathetic nerve terminals. *Br. J. Pharmac.*, **42**, 263–272.

VIZI, E.S. (1968). The inhibitory effect of noradrenaline and adrenaline on release of acetylcholine from guinea-pig ileum longitudinal strips. *Naunyn Schmiedeberg's Arch. exp. Path. Pharmak.*, **259**, 199–200.

WEETMAN, D.F. (1972). The guinea-pig isolated innervated bladder preparation: the effects of some autonomic drugs. *Arch. int. Pharmacodyn*, **196**, 383–392.

WEETMAN, D.F. & TURNER, N. (1973). The effect of ganglion blocking agents on the isolated, innervated bladder of the guinea-pig. *Arch. int. Pharmacodyn*, **201**, 100–105.

*Br. J. Pharmac.* (1976), **58**, 43–46

# CIRCULATORY EFFECTS OF ORAL AND SUBCUTANEOUS ADMINISTRATION IN NORMAL SUBJECTS OF A NEW BRONCHODILATOR, IBUTEROL, A PRO-DRUG TO TERBUTALINE

ELIN HÖGLUND, H. WESTLING & T. WHITE
Department of Clinical Physiology, University of Lund, Lasarettet, S-221 85 Lund, Sweden

1 The di-isobutyryl ester of terbutaline, ibuterol, was given subcutaneously and orally to healthy volunteers and its effects on heart rate, arterial blood pressure, and ECG were compared with those of terbutaline itself. The effects of the two substances were qualitatively identical, but quantitatively different.

2 By mouth, ibuterol acted more rapidly, and was 2–3 times more active. After intramuscular injection, ibuterol caused less increase in local blood flow than terbutaline.

3 Ibuterol can be regarded as a pro-drug, which in itself has little activity, but which can be made active in the body.

## Introduction

Modern sympathomimetic bronchodilators are active by mouth and cause fewer cardiac side effects in relation to their bronchodilator effects. The best known of these drugs are terbutaline and salbutamol (Carlström, 1970; Lewis, 1971). Terbutaline has to be given in much larger doses by mouth to produce the same effects as a subcutaneous injection (Arner, Bertler, Karlefors & Westling, 1970). The di-isobutyryl ester of terbutaline (ibuterol) is hydrolyzed in the body of experimental animals to the active drug terbutaline (Olsson, Persson, Persson & Sörenby, 1974).

## Methods

### *Drugs*

Terbutaline was used as the sulphate, molecular weight 549 (equivalent weight 274) and ibuterol as the hydrochloride, molecular weight 402. The ratio between equivalent doses of ibuterol hydrochloride and terbutaline sulphate is 1.47. Both drugs were given in 50 ml water by the mouth, and in 0.5 ml sterile 0.9% w/v NaCl solution subcutaneously. Ibuterol and terbutaline (Bricanyl) were supplied by AB Draco, Sweden (a subsidiary of AB Astra, Sweden).

### *Subjects*

Eight healthy male volunteers were informed of the nature of the study and received payment. Four of the subjects were used in experiments on the effects of drugs on heart rate, blood pressure, and ECG. In the remaining four subjects muscle blood flow was measured.

### *Procedures*

*Measurements of heart rate, blood pressure, and ECG.* The subjects were fasting, and smoking was not allowed before the test. They sat in a reclining chair. Heart rate was measured from a continuous ECG (CR chest leads); T wave amplitude was measured in lead CR5. Arterial arm blood pressure was measured by the auscultatory method. Baseline values were obtained at the end of a 60 min resting period. Observations were made at 5 or 15 min intervals for 240 min after the drug (or placebo). A light meal (bread, butter, milk) was given 180 min after the drug. Subjective symptoms were recorded. Average basal values for heart rate were 48–61 beats/minute. There were no significant differences between basal values on different days in each individual.

Each of the four subjects was first given 4 drug treatments by mouth at one week intervals, 5 and 10 mg of terbutaline sulphate and of ibuterol hydrochloride (Latin square design). Thereafter the effects of subcutaneous administration of 0.5 and 1.0 mg terbutaline sulphate and 1.25 and 2.5 mg ibuterol hydrochloride were studied in the same way.

*Measurement of muscle blood flow.* Blood flow in the anterior tibial muscle was measured by the

$^{133}$xenon wash-out technique (Lindbjerg, 1965; Arner *et al.*, 1970). In each of the four subjects muscle blood flow was studied four times in both legs, and each subject received two doses of terbutaline sulphate and two doses of ibuterol hydrochloride, so that eight observations were made on each drug dose.

### *Statistical analysis*

Analysis of variance, and '4 point assay' for determination of relative potency were used. The total effect on the heart rate was calculated as the area of increased heart rate over 3 and 4 hours.

## Results

### *Qualitative effects of ibuterol*

Tachycardia, increase in systolic blood pressure and pulse pressure, and decrease in T wave amplitude were regularly seen. The systolic blood pressure returned more rapidly towards the baseline than the heart rate (Figures 1 and 2). The patients often reported palpitations and tremor, particularly after the higher doses of the drugs. The circulatory effects of the meal were similar to those of the smaller drug doses (Figure 1).

### *Activity of ibuterol in relation to terbutaline; subcutaneous injection*

Both drugs acted within 5 min and gave peak heart rates at 30–60 min (Figure 2). Thereafter, the effects of terbutaline declined more rapidly, but even after 180 min the heart rate was still increased. The effects of ibuterol lasted slightly longer. On a molar basis ibuterol was less active than terbutaline; the equiactive doses being related approximately 1.5 : 1 (Table 1).

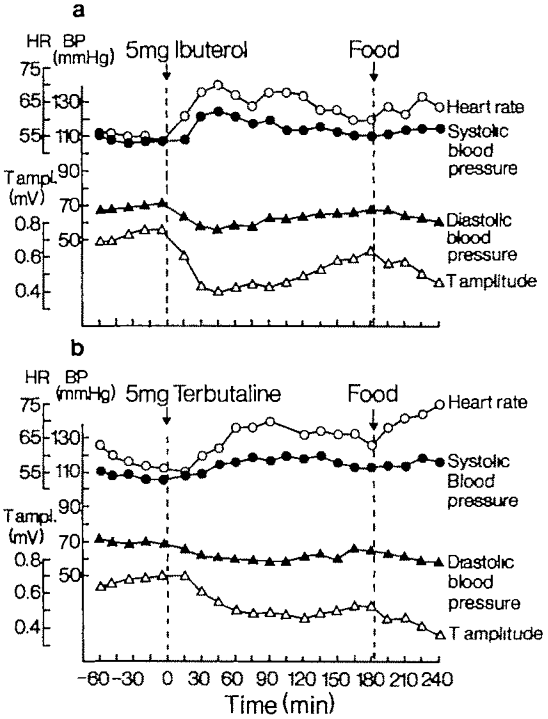


**Figure 1** Mean values for heart rate (beats/min), arterial blood pressure (1 mmHg ≈133 Pa) and T wave amplitude after oral administration of (a) 5 mg ibuterol, (b) 5 mg terbutaline. Drugs were given at 0 min and a light meal just after the observation at 180 minutes.

### *Activity of ibuterol in relation to terbutaline; oral administration*

Ibuterol acted more rapidly and caused significant increase in heart rate in less than 15 min whereas

**Table 1** Ratio between equiactive doses of ibuterol and terbutaline

| *Ratio* | *Maximal increase in heart rate* | *Total increase in heart rate* | |
|---|---|---|---|
| *Equiactive doses* | | *over 3 h* | *over 4 h* |
| Ibuterol s.c.<br>Terbutaline s.c. | 1.5<br>(1.27–1.92) | 1.4<br>(1.15–1.67) | 1.4<br>(1.12–1.68) |
| Terbutaline orally<br>Terbutaline s.c. | 15<br>(11.6–18.5) | 13<br>(10.5–1.67) | 11<br>(9.2 –14.1) |
| Ibuterol orally<br>Ibuterol s.c. | 5.2<br>(4.1–6.5) | 5.0<br>(4.0–6.3) | 4.9<br>(3.9–6.0) |

The ratios are calculated on a molar basis and based on changes in heart rate expressed in three different ways. Mean values (and 95% confidence limits) are given.

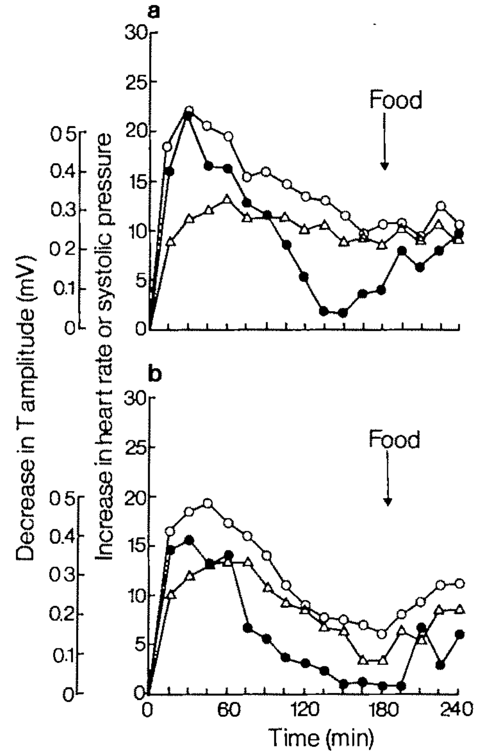


**Figure 2** Mean changes in heart rate (O, beats/min), systolic blood pressure (●, mmHg; 1 mmHg ≈ 133 Pa) and T wave amplitude (Δ) after subcutaneous administration of (a) 1.25 mg ibuterol, (b) 0.5 mg terbutaline. Drugs were given at 0 min and a light meal just after the observation at 180 minutes.

terbutaline acted only after a delay of 15–30 min (Figure 1). In addition the maximal effects of terbutaline occurred later. There were similar differences for arterial blood pressure and T wave amplitude.

Table 1 shows the relative potency of the two drugs. The effectiveness by mouth, as compared to that after subcutaneous administration, was greater for ibuterol than for terbutaline.

### *Direct effects of ibuterol and terbutaline on muscle bloodflow*

Resting blood flow without drugs under the present experimental conditions was 1 to 4 ml blood min$^{-1}$ 100 ml tissue$^{-1}$. Table 2 shows that 0–2 min after injection, a 60 times greater concentration of ibuterol than of terbutaline was required to produce the same blood flow. Clearly, ibuterol was a less potent vasodilator than terbutaline. Physostigmine (to a final concentration of 10 µg/ml) did not change the responses to ibuterol or terbutaline appreciably. This suggests absence of local hydrolysis of ibuterol (cf. Olsson *et al.*, 1974).

## Discussion

With both ibuterol and terbutaline the pressor effect waned earlier than the tachycardia. The phenomenon may indicate differences in adaptation between receptors (or mechanism) responsible for the cardiovascular actions of the drugs (cf. Conolly, Davies, Dollery & George, 1971).

Ibuterol is slightly less active than terbutaline after subcutaneous injection. A similar difference was observed in animal experiments (Olsson *et al.*, 1974) and may be due to incomplete hydrolysis of ibuterol.

By mouth ibuterol was more effective, and also acted faster than terbutaline. These differences may be related to the different physicochemical properties of the two drugs which could influence the site or mode of absorption. Ibuterol is lipophilic and terbutaline is hydrophilic.

Ibuterol can be regarded as a pro-drug to terbutaline (Review notice, 1974). The greater oral efficiency of ibuterol may give more rapid and predictable therapeutic effects (Arner & Magnusson, 1976).

**Table 2** Muscular blood flow in response to ibuterol and terbutaline added locally

| *Drug* | *Concentration (µg/ml)* | *Blood flow (ml min$^{-1}$ 100 ml$^{-1}$)* | |
|---|---|---|---|
| | | *0–2 min* | *3–5 min* |
| Ibuterol hydrochloride | 12.5 | 14.0 ± 2.5 | 14.0 ± 1.2 |
| Ibuterol hydrochloride | 200 | 20.4 ± 1.7 | 23.1 ± 2.3 |
| Terbutaline sulphate | 3.125 | 20.0 ± 2.5 | 18.6 ± 2.2 |
| Terbutaline sulphate | 50 | 30.0 ± 4.6 | 29.8 ± 2.5 |

Average values ± s.e. mean are given for eight observations immediately (0–2 min) and 3–5 min after injection. Note that the injected volume was 0.2 ml and consequently the doses of drugs varied from 0.625 to 40 µg.

## References

ARNER, B., BERTLER, Å., KARLEFORS, T. & WESTLING, H. (1970). Circulatory effects of orciprenaline, adrenaline and a new sympathomimetic $\beta$-receptor-stimulating agent, terbutaline, in normal human subjects. *Acta medica scand.*, Suppl. **512**, 25–32.

ARNER, B. & MAGNUSSON, P.O. (1976). Comparison between ibuterol hydrochloride and terbutaline in asthma. *Br. med. J.*, **1**, 72–74.

CARLSTRÖM, S. ed. (1970). Studies on terbutaline, a new selective bronchodilating agent. *Acta med. scand.*, Suppl. **512**, 1–48.

CONNOLLY, M.E., DAVIES, D.S., DOLLERY, C.T. & GEORGE, C.F. (1971). Resistance to $\beta$-adrenoceptor stimulants (a possible explanation for the rise in asthma deaths). *Br. J. Pharmac.*, **43**, 389–402.

LEWIS, A.A.G. (1971). Salbutamol. *Postgraduate med. J.*, Suppl. **47**, 1–133.

LINDBJERG, I.F. (1965). Measurement of muscle blood flow with $^{133}$Xe after histamine injection as a diagnostic method in peripheral arterial disease. *Scand. J. Clin. Lab. Invest.*, **17**, 371–380.

OLSSON, O.A.T., PERSSON, C.G.A., PERSSON, H. & SÖRENBY, L. (1974). Pharmacological properties of 1-(3′ 5′-diisobutyryloxyphenyl)-2-(*t*-butylamino)-ethanohydrochloride (KWD 2058), a new sympathomimetic bronchodilator. *Acta pharmac. tox.*, **35**, 76–90.

REVIEW NOTICE (1974). *Chemical and Engineering News*, **52**, 26–27.

*(Received October 9, 1975.*
*Revised March 1, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 47–55

# THE ACTION OF TETRAETHYLAMMONIUM CHLORIDE ON THE RESPONSE OF THE RAT ANOCOCCYGEUS MUSCLE TO MOTOR AND INHIBITORY NERVE STIMULATION AND TO SOME DRUGS

J.S. GILLESPIE & A.K. TILMISANY
Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

**1** Tetraethylammonium chloride (TEA) 0.125 mM to 20 mM potentiates the response of the anococcygeus muscle to field stimulation of the motor adrenergic nerves without affecting the response to noradrenaline suggesting a pre-synaptic origin of potentiation. The potentiation is greatest at low, submaximal, frequencies (2 Hz) of stimulation and only slight at the higher frequency of 20 Hz. This difference is due to the restraint imposed on the demonstration of potentiation by maximal or near maximal motor responses since reduction of the mechanical response at 20 Hz by either phentolamine (post-synaptic block) or guanethidine (pre-synaptic block) resulted in a great increase in potentiation of the response at this frequency.

**2** TEA in concentrations up to 1 mM similarly potentites the response to inhibitory nerve stimulation and again the greatest effect is at low frequencies. Higher concentrations (5–20 mM) progressively depress the inhibitory response. It is suggested that TEA may specifically antagonize the post-synaptic action of the inhibitory transmitter and that at higher concentrations of TEA this effect dominates the pre-synaptic action in increasing transmitter release.

**3** TEA has no effect on the motor response to tyramine.

**4** TEA (5–20 mM) causes a maintained rise in muscle tone. Part of this is abolished by phentolamine but part is resistant. A similar muscle stimulant action of TEA is observed in muscles from rats previously treated with 6-hydroxydopamine in which indirect sympathomimetic drugs and field stimulation could no longer produce a motor response. These results suggest that part of the motor effect of TEA is due to an increased spontaneous release of noradrenaline and part to a direct action on the muscle.

**5** TEA 0.125 mM to 20 mM antagonize the stimulant action of carbachol. Dose-response curves show a parallel shift to the right with no change in the maximum response suggesting a competitive atropine-like action. Such an effect has previously been reported in amphibian tissue but not so far as we can determine in mammalian preparations.

**6** The possible mode of action of TEA is discussed.

## Introduction

Tetraethylammonium chloride (TEA) possesses a variety of actions which result from its interference with cholinergic transmission. In addition to the effects at acetylcholine receptors, TEA has important effects on the ion permeability of the membranes of many excitable cells. One particular effect is to prolong the action potential in muscle and nerve by preventing the delayed rise in potassium permeability which contributes to the rapid restoration of the resting membrane potential (Fatt & Katz, 1953; Tasaki & Hagiwara, 1957; Armstrong & Binstock, 1965). This prolongation of the action potential increases the liberation of transmitter from both cholinergic (Kensler, 1950; Koketsu, 1958; Douglas & Lywood, 1961; Collier & Exley, 1963) and adrenergic nerves (Thoenen, Haefely & Staehelin, 1967; Kirpekar, Prat, Puig & Wakade, 1972; Stjarne, 1973).

The experiments described in this paper were done to determine whether TEA would increase the release of transmitter from the nerves to the anococcygeus muscle and, in particular, whether it would potentiate the response to stimulation of the inhibitory nerves, a non-cholinergic non-adrenergic neurone whose transmitter is at present unknown (Gillespie, 1972). A preliminary account of some of these results has been published (Gillespie & Tilmisany, 1974).

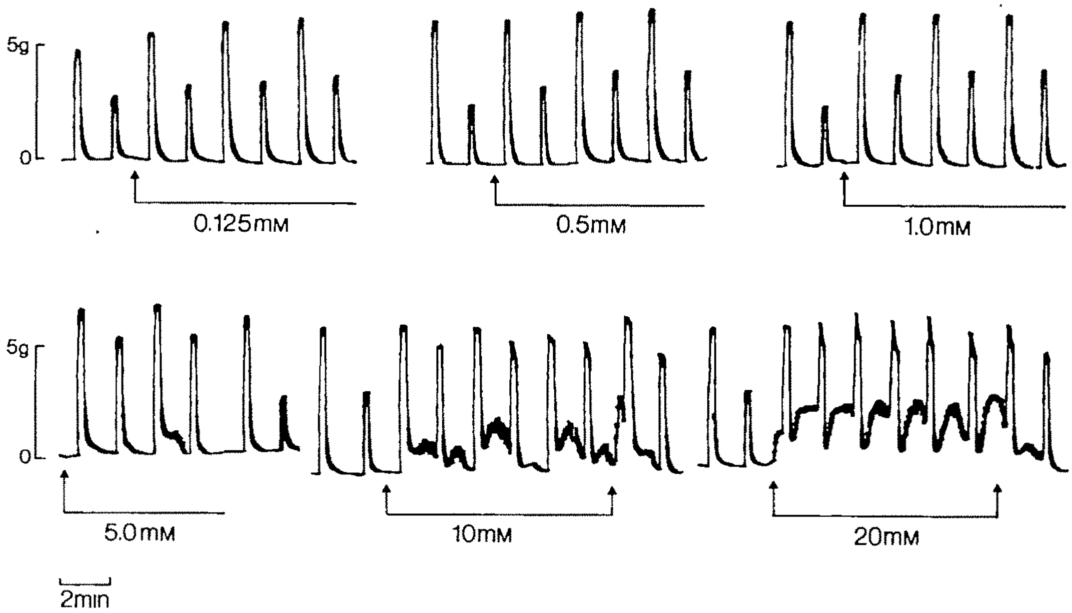


**Figure 1** The effect of increasing concentrations of tetraethylammonium (TEA) on the motor response of the rat anococcygeus muscle to field stimulation. The preparation was stimulated alternatively at 20 and 2 Hz; TEA in the concentration shown below each trace was added at the arrows. TEA potentiated the motor response at both 20 and 2 Hz but the effect was greater at 2 Hz so that at a concentration of 20 mM TEA the responses at both frequencies became equal. TEA 10 and 20 mM also caused a sustained rise in muscle tone. In this and all subsequent figures the amplitude of the motor response was measured from the level of tone existing before the addition of TEA.

## Method

Rat anococcygeus muscles were isolated and suspended in 20 ml baths of Krebs saline at 37°C and gassed with a mixture of 95% $O_2$ and 5% $CO_2$ as previously described (Gillespie, 1972). Only male animals were used since in this species the muscles are thicker and stronger in the male and variability is reduced by using only one sex. The muscles were passed through platinum ring electrodes for field stimulation. Stimulation was by 1 ms pulses at supramaximal voltage and at the frequencies shown in the text. The tissue was stimulated for fixed periods of 10–20 s repeated at intervals varying in different experiments from 100 s to 3 minutes. The interval between periods of stimulation and the duration of stimulation were preset by appropriate timing devices. Drugs were added to the bath dissolved in 0.9% w/v NaCl solution (Saline) in volumes of 0.1 to 0.3 ml.

Drugs used were tetraethylammonium bromide (Koch-Light), carbachol (Sigma), guanethidine sulphate (Ciba), (—)-noradrenaline bitartrate (Koch-Light), hexamethonium bromide (Koch-Light), phentolamine mesylate (Ciba), atropine sulphate (BDH), (+)-tubocurarine (Burroughs Wellcome), 6-hydroxydopamine (Calbiochem) and tyramine (Sigma). Doses are given as moles of the base.

## Results

### *The effect of TEA on the motor response to field stimulation*

TEA in concentrations of 0.125 mM and higher potentiated the motor response to field stimulation (Figure 1) with little or no potentiation of the response to noradrenaline (Figure 4). The degree of potentiation of the response to field stimulation varied with frequency and with the concentration of TEA. Potentiation was most obvious at low frequencies and was relatively slight at the optimum frequency of stimulation of 20 Hz (Figure 1, Table 1). As a result, the effect of the drug was to make the response at 2 Hz equal to that at 20 Hz. Increasing the concentration of TEA increased the degree of potentiation up to at least a concentration of 20 mM (Table 1). Estimation of the effect of TEA concentrations greater than 5 mM was complicated by the maintained rise in

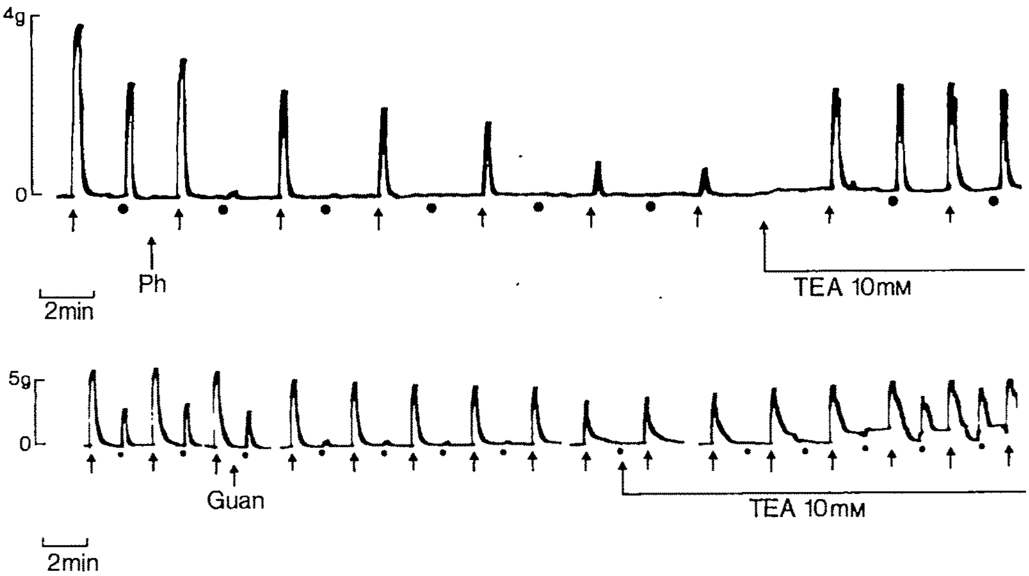


**Figure 2** The effect of tetraethylammonium (TEA) 10 mM on the motor response of the rat anococcygeus to field stimulation in the presence of either phentolamine (Ph 20 µM) or guanethidine (Guan, 10 µM). The records are from two different muscles. In both the blocking agent reduces the motor response at 20 Hz (▲) and completely abolishes that at 2 Hz (●); TEA restores the response at both frequencies.

muscle tone which these concentrations induced (Figure 1) so that concentrations above 20 mM were not investigated.

The smallness of the mean potentiation at 20 Hz (see Table 1) could have been due to a failure of TEA to increase transmitter output at this frequency or to the fact that the muscle response was already near maximal and little or no increase in tension was possible whatever the effect on transmitter output. These possibilities were distinguished by the use of blocking drugs to impair transmission and produce a submaximal response at 20 Hz suitable for the detection of potentiation. Two blocking agents were separately used, phentolamine 2 µM to produce post-synaptic α-adrenoceptor blockade and guanethidine 1 µM to produce pre-synaptic blockade. The results are shown in Figure 2. TEA reversed both pre- and post-synaptic block.

**Table 1** The mean potentiation or inhibition of the motor response to field stimulation produced by tetraethylammonium (TEA) at various concentrations

| *Concentration of TEA mM* | *Potentiation or inhibition as a % of the maximum response* | | |
|---|---|---|---|
| | *50 Hz* | *20 Hz* | *2 Hz* |
| 0.125 | 2.1 ± 1.8 | 6.4 ± 2.5* | 8.7 ± 2.7** |
| 0.25 | 3.7 ± 1.9 | 8.3 ± 3.6* | 9.6 ± 6.7*** |
| 0.5 | −2.4 ± 2.9 | 7.7 ± 2.7* | 16.7 ± 2.7*** |
| 1.0 | −4.6 ± 5.5 | 6.2 ± 2.2** | 22.3 ± 3.6*** |
| 5.0 | −7.7 ± 4.3 | 5.2 ± 2.4* | 40.8 ± 4.9*** |
| 10.0 | −3.1 ± 5.9 | 9.2 ± 2.4*** | 47.6 ± 4.4*** |
| 20.0 | −10.2 ± 5.0 | 14.1 ± 2.4*** | 54.4 ± 4.7*** |

In each experiment potentiation or inhibition was measured as the increase or decrease in the response compared with the control at that frequency and expressed as a percentage of the maximum response the tissue could produce. The amplitude of the motor response was measured from the level of tone existing before the addition of TEA. The values in the table are the means ± s.e. mean and the number of muscles tested varied from 6 to 39.

The significance of the results was assessed by Student's *t* test; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

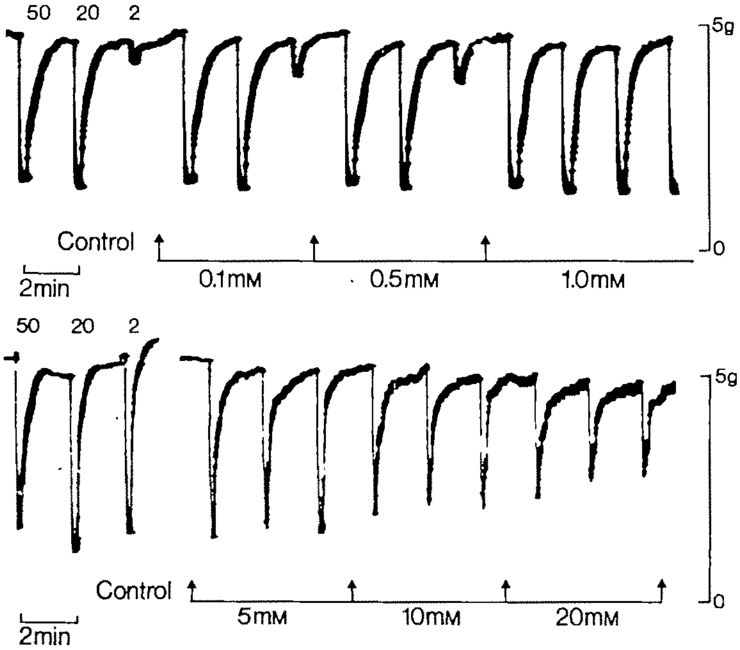


**Figure 3** The effect of increasing concentrations of tetraethylammonium (TEA) on the inhibitory response of the rat anococcygeus muscle to field stimulation at 50, 20 or 2 Hz. The upper and lower records are from two different muscles; the concentrations of TEA are shown below the records. Guanethidine (30 μM) was present to block the motor response and raise tone. Concentration of TEA up to 1 mM potentiated the inhibitory response especially at 2 Hz; higher concentrations of 10 and 20 mM reduced both amplitude and duration of the response.

### *The effect of TEA on the inhibitory response to field stimulation*

In these experiments guanethidine 30 μM was added to the bath to block the motor adrenergic nerves and simultaneously, by an indirect sympathomimetic action, to raise tone. It is on the background of this raised tone that the presence of an inhibitory innervation can be demonstrated. TEA in concentrations from 0.1 to 1 mM potentiated the inhibitory response to field stimulation and once again the effect was most marked with the lower frequencies of stimulation (Figure 3) so that the response to 2 Hz was increased so as to equal the maximum response. Higher concentrations of TEA reduced the inhibitory response both in amplitude and duration (Figure 3).

### *The effect of TEA on the response to tyramine*

Transmitter may be liberated from an adrenergic nerve either spontaneously, in response to the arrival of an action potential, or by displacement from storage sites by indirect sympathomimetic drugs. The first two mechanisms require calcium, the last does not and it was therefore of interest to see whether transmitter liberation by an indirect sympathomimetic agent would be affected by TEA. As Figure 4 shows, TEA in concentrations which potentiate the response to nerve stimulation had no effect on the response to tyramine.

### *Direct effects of TEA*

Concentrations of TEA greater than 5 mM caused a maintained rise in tone in the preparation (Figure 5). The possibility that this was due to an increased spontaneous release of noradrenaline was investigated by studying the effect of phentolamine. Part, but not all, of the rise in tone was abolished by phentolamine 10 μM confirming that part of the effect was due to the release of noradrenline but that there was in addition a direct action on the smooth muscle. This was further investigated by treating four rats with 6-hydroxydopamine (6-OHDA) to destroy the adrenergic nerve terminals and then examining the effect of TEA. Examination of these muscles with the Falck fluorescence technique showed no fluorescent adrenergic nerves and the muscles, though responding

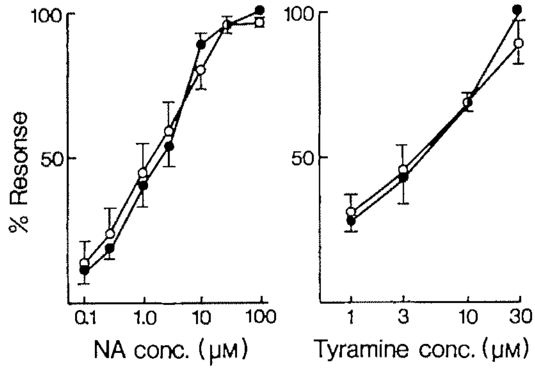


**Figure 4** Dose-response curves of the rat anococcygeus muscle to noradrenaline (NA) and tyramine in the presence (O) and absence (●) of tetraethylammonium (TEA) 1 mM. In each experiment responses are expressed as a percentage of the maximum response in that experiment. Each point is the mean of observations and the vertical lines represent s.e. mean. TEA had no significant effect on the response to either agonist.

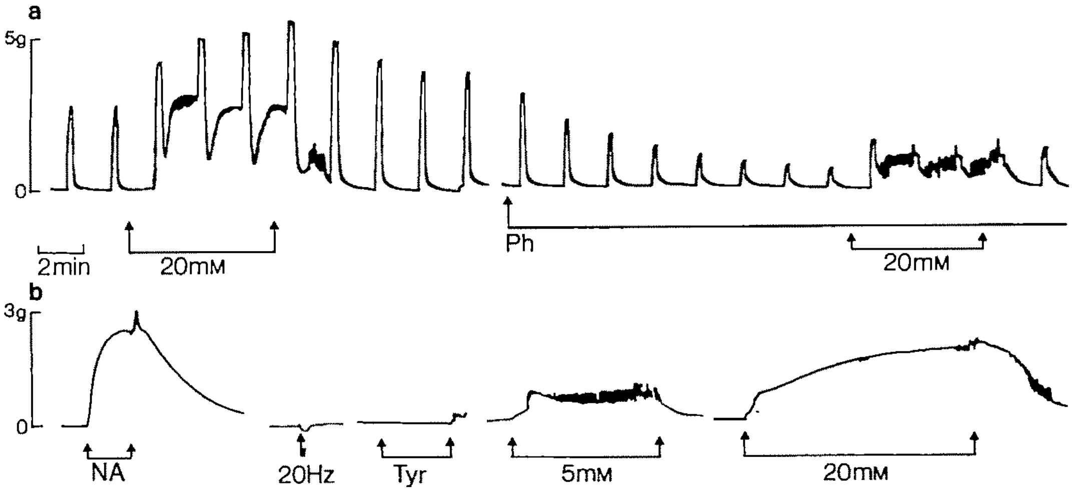


**Figure 5** (a) The effects of tetraethylammonium (TEA) 20 mM in potentiating the motor response to field stimulation at 20 Hz and also causing a considerable rise in tone in a rat anococcygeus muscle. Phentolamine (Ph 10 μM) almost blocked the response to field stimulation and greatly reduced but did not abolish the rise in tone produced by TEA. (b) Records from a muscle from a rat previously treated with 6-hydroxydopamine to destroy the adrenergic nerves. The muscle was still sensitive to noradrenaline (NA, 3 μM) but neither field stimulation nor tyramine (Tyr, 10 μM) caused contraction. TEA 5 mM and 20 mM still produced a rise in tone.

to noradrenaline, gave no motor response to either field stimulation or the indirect sympathomimetic agent tyramine; nonetheless, TEA 5 and 20 mM produced a graded contraction (Figure 5).

### *Atropine-like action of TEA*

In the experiments described in the previous paragraph the continuing presence of an intact inhibitory innervation in tissues from 6-OHDA-treated animals was checked by raising tone with carbachol since guanethidine was ineffective in these circumstances. It was found that TEA abolished this carbachol-induced tone. This was further investigated in muscles from normal animals and, as Figure 6 shows, TEA was able to abolish the effects of carbachol in a dose-related fashion. Dose-response curves of rise in tone for carbachol in the presence of

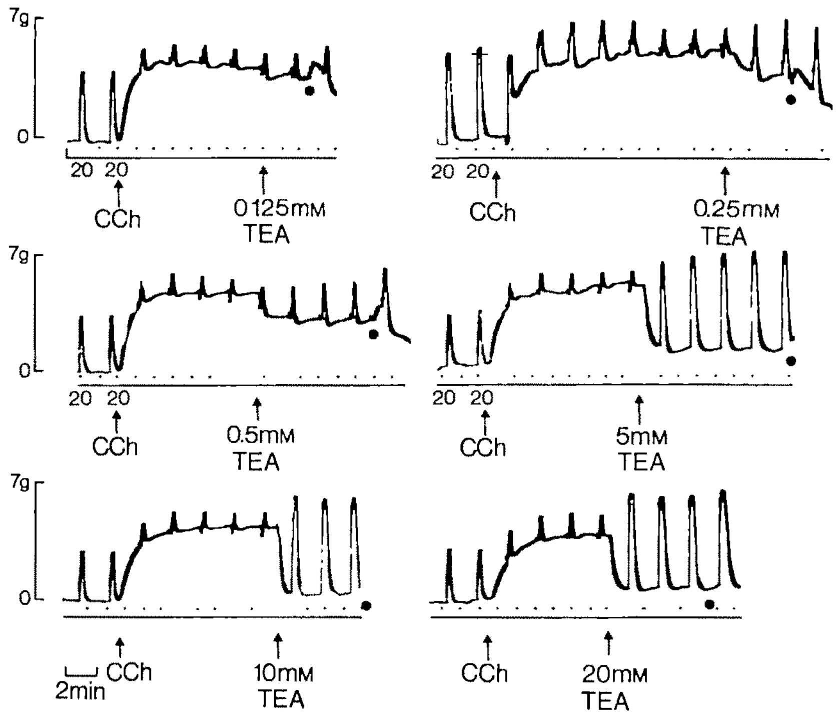


**Figure 6** The effect on the rat anococcygeus muscle of increasing concentrations of tetraethylammonium (TEA) on the increase in muscle tone induced by carbachol (CCh, 5 μM). Field stimulation at 20 Hz was applied at 2 min intervals. The lowest concentration of TEA, 0.125 M, slightly lowered tone, an effect which increased with concentration up to a maximum at 10 mM. The response to field stimulation was also potentiated. At the black dot the bath was washed.

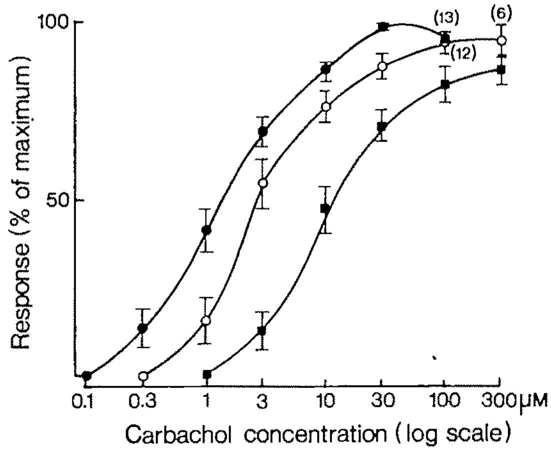


**Figure 7** Mean dose-response curves of rise in tone to carbachol alone (●), and in the presence of tetraethylammonium (TEA) 0.25 mM (O) and 1.0 mM (■). Responses are expressed as a percentage of the maximum response to carbachol in the control. Number of muscles was 15 for all concentrations unless otherwise indicated. The vertical lines represent s.e. mean.

0.25 and 1.0 mM TEA are illustrated in Figure 7 and shows a parallel shift of the dose response curves with no change in the maximum response, consistent with a competitive blocking effect.

### *The mode of action of TEA*

Adrenergic nerves are known to possess a variety of pre-synaptic receptors capable of feedback inhibition of transmitter release. Prominent among these are nicotinic and muscarinic acetylcholine receptors and the α-receptor for noradrenaline itself. We, therefore, investigated whether block of nicotinic receptors by hexamethonium or curare, of muscarinic receptors by atropine or of α-receptors by phentolamine would abolish the ability of TEA to cause potentiation. The results are shown in Figure 8. None of these blocking agents prevented either the potentiation of the response to motor nerve stimulation or the effect of TEA in raising muscle tone.

## Discussion

TEA produces a variety of effects in the anococcygeus muscle. The potentiation of the response to both

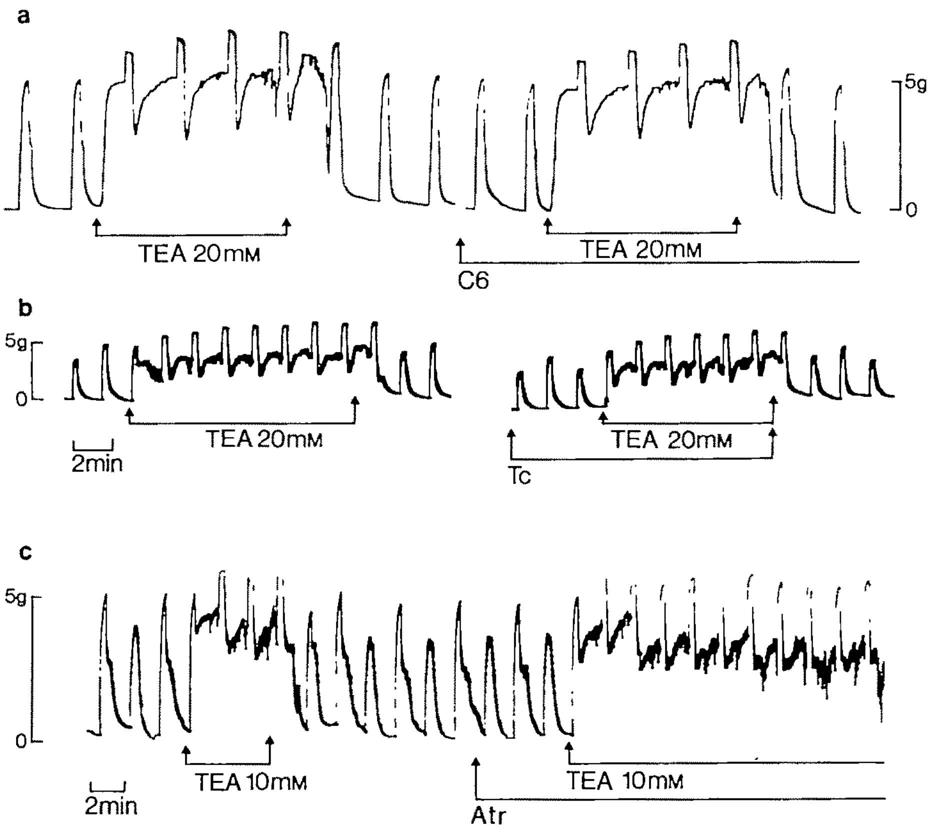


**Figure 8** The responses of three different anococcygeus muscles to field stimulation and to tetraethylammonium (TEA) 20 mM and the effect on these of (a) hexamethonium (C6, 10 μM). (b) (+)-tubocurarine (Tc, 3 μM) and (c) atropine (Atr, 10 μM). The muscle in (a) was stimulated intermittently at 20 Hz. The muscles in (b) and (c) were stimulated at 2 and 20 Hz alternately. Neither the potentiation of the response to field stimulation nor the rise in muscle tone was affected by these blocking drugs.

motor and inhibitor nerve stimulation is almost certainly due to an increased liberation of their respective transmitters as a result of prolongation and augmentation of the nerve action potential and a consequent increased entry of calcium. Similar concentrations of TEA have been shown to prolong the action potential in muscle (Fatt, & Katz, 1953; Hagiwara & Watanabe, 1955) and nerve (Schmidt & Stämpfli, 1966; Taski & Hagiwara, 1957) and to increase transmitter liberation from cholinergic and adrenergic nerves (see earlier references). The lack of potentiation of the response to tyramine is in agreement with this explanation since calcium is not involved in the release of transmitter by this drug.

The phentolamine-sensitive rise in tone with concentrations of TEA above 5 mM is probably due to a direct liberation of noradrenaline by TEA. Such concentrations are likely to cause depolarization and instability of the terminal nerve membrane and this instability alone would increase the spontaneous liberation of transmitter. This instability might be sufficient to induce spontaneous action discharge but previous work suggests that higher concentrations of TEA are normally necessary for this (Beaulieu & Frank, 1967a). The phentolamine-resistant component of the stimulant action of TEA suggests an additional, direct, action on the smooth muscle. TEA has been reported to stimulate other smooth muscles; the guinea-pig ileum (Collins, 1948; Beleslin & Rakič, 1969, the guinea-pig stomach (Ito, Kuriyama & Sakamoto, 1970) and the bovine trachea (Kirkpatrick, 1975. The probable mechanism suggested by Kirkpatrick (1975) and Ito *et al.* (1970) is that TEA abolishes the rectifying properties of the muscle membrane by reducing the normally high potassium permeability. The result is some depolarization but more dramatically an instability of the membrane so that, for example, muscles previously incapable of generating spike potentials can now do so. Consistent with such an explanation is the finding that the anococcygeus normally has a stable membrane potential, rarely generates spike potentials and these never show overshoot. In the presence of TEA the muscle is depolarized and generates large spike potentials with several millivolts of overshoot (Creed, Gillespie & Muir, 1975). It is only to be expected that in such circumstances the muscle would develop spontaneous tone.

We found little or no potentiation of the response to noradrenaline. In the cat spleen (Thoenen *et al.*, 1967) and the rabbit pulmonary artery (Bevan, 1963), TEA in addition to increasing transmitter release caused a two- to six-fold increase in sensitivity to noradrenaline. The lack of potentiation in the experiments described here is all the more surprising in view of the direct depolarizing effect of TEA on the muscle and the appearance of spikes with overshoot; effects which would be expected to be synergistic with any other depolarizing agonist. However, other workers have reported a failure of TEA to potentiate some agonists while markedly augmenting others on the same tissue (Collins, 1948).

TEA reversed the blocking action of both phentolamine and guanethidine; a similar reversal of guanethidine block by TEA has been reported in the guinea-pig vas deferens (Maanen & Werty, 1966). An increased release of transmitter with more effective competition with phentolamine would be a sufficient explanation of the reversal of post-synaptic block by TEA. The mechanism of guanethidine reversal is less obvious. It may be the augmented nerve action potential allows entry of calcium over a longer period and the resulting increase in neurosecretory coupling efficiency together with the post-synaptic hypersensitivity characteristic of guanethidine is sufficient explanation. A more interesting alternative is that the antagonism is more specifically related to the mode of action of guanethidine and involves an increase in the mobility of calcium within the membrane as suggested by Beaulieu & Frank (1967b). A similar hypothesis might account for the reduction by higher concentrations of TEA of the inhibitory response to field stimulation. Beaulieu & Frank (1967b) have called attention to the paradoxical effects of TEA which produces membrane instability in muscle and nerve in a way reminiscent of low calcium, yet appears able to potentiate the effects of calcium in, for example, the release of neurotransmitter or the restoration of potassium contractures previously abolished by low calcium. Beaulieu & Frank (1976b) suggest both phenomena are the result of a single action of TEA, the displacement of calcium from binding sites in the membrane. This causes membrane instability but at the same time the displaced calcium is made available within the cytoplasm for excitation-contraction or excitation-secretion coupling. If this explanation is correct then the mode of action of the inhibitory transmitter may be to increase calcium binding to the membrane, and TEA may be a relatively specific antagonist.

One other action of TEA was to inhibit the response to carbachol in a competitive fashion suggesting an atropine-like action on muscarinic receptors. Among the earliest work on TEA are reports of just such an effect (Schüller, 1920; Külz, 1922). However, these experiments were on amphibian tissue (frog rectum, frog heart) and a similar atropine-like action on the blood pressure and pupil of the cat could not be confirmed (Hunt & Renshaw, 1925). We have been unable to find convincing reference in the literature of an atropine-like action in the mammal. It may be that pre-existing parasympathetic tone potentiated by TEA has obscured the post-synaptic blocking effect just as at the skeletal neuromuscular junction the pre-synaptic, anti-curare action can obscure the curare-like block by TEA.

Finally, the site of action of TEA should be considered. TEA penetrates cells only with difficulty and when introduced by iontophoresis its effects are slow to develop and difficult to reverse (Kleinhaus & Prichard, 1975). All of the effects reported in this paper were rapid in onset and easily and completely reversed by washing, suggesting the effects were exerted on the outside of the various cell membranes.

We are grateful to the University of Riyadh for a grant to one of us (A.K.T.) and to the Rankin Fund of Glasgow University for a grant for apparatus.

## References

ARMSTRONG, C.M. & BINSTOCK, L. (1965). Anomalous rectification in squid giant axon injected with tetraethylammonium chloride. *J. gen. Physiol.*, **48**, 859–886.

BEAULIEU, G. & FRANK, G.B. (1967a). Tetraethylammonium-induced contractions of frog's skeletal muscle. II. Effects on intramuscular nerve endings. *Can. J. Physiol. Pharmac.*, **45**, 833–844.

BEAULIEU, G. & FRANK, G.B. (1967b). Tetraethylammonium-induced contractions of frog's skeletal muscle. III. Mechanism of action by calcium release. *Can. J. Physiol. Pharmac.*, **45**, 845–855.

BELESLIN, D.B. & RAKIČ, M.M. (1969). Stimulant action of tetraethylammonium on the persitaltic reflex of the guinea-pig isolated ileum. *Br. J. Pharmac.*, **37**, 245–250.

BEVAN, J.A. (1963). Action of tetraethylammonium chloride on the sympathetic ganglia-pulmonary artery preparation. *J. Pharmac. exp. Ther.*, *140*, 193–198.

COLLIER, B. & EXLEY, K.A. (1963). Mechanism of the antagonism by tetraethylammonium of neuromuscular block due to d-tubocurarine or calcium deficiency. *Nature, Lond.*, **199**, 702–703.

COLLINS, D.A. (1948). Influence of tetraethylammonium on responses of isolated intestine to angiotensin and other substances. *J. Pharmac. exp. Ther.*, **94**, 244–248.

CREED, K.E., GILLESPIE, J.S. & MUIR, T.C. (1975). The electrical basis of excitation and inhibition in the rat anococcygeus muscle. *J. Physiol., Lond.*, **245**, 33–47.

DOUGLAS, W.W. & LYWOOD, D.W. (1961). The stimulant effect of TEA on acetylcholine output from the superior cervical ganglion: a comparison with barium. *Fedn Proc.*, **20**, 324.

FATT, P. & KATZ, B. (1953). The electrical properties of crustacean muscle fibres. *J. Physiol., Lond.*, **120**, 171–204.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmac.*, **45**, 404–416.

GILLESPIE, J.S. & TILMISANY, A.K. (1974). The effect of tetraethylammonium (TEA) on the anococcygeus muscle and on its response to motor and inhibitory nerve stimulation. *Br. J. Pharmac.*, **51**, 141P.

HAGIWARA, S. & WATANABE, A. (1955). The effect of tetraethylammonium chloride on the muscle membrane examined with an intracellular microelectrode. *J. Physiol., Lond.*, **129**, 513–527.

HUNT, R. & RENSHAW, R.R. (1925). On some effects of arsonium, stibonium, phosphonium and sulfonium compounds on the autonomic nervous system. *J. Pharmac.*, **25**, 315–355.

ITO, Y., KURIYAMA, H. & SAKAMOTO, Y. (1970). Effects of tetraethylammonium chloride on the membrane activity of guinea-pig stomach smooth muscle. *J. Physiol., Lond.*, **211**, 445–460.

KENSLER, CH. J. (1950). The anticurare activity of tetraethylammonium ion in the cat. *Br. J. Pharmac. Chemother.*, **5**, 204–209.

KIRKPATRICK, C.T. (1975). Excitation and contraction in bovine tracheal smooth muscle. *J. Physiol., Lond.*, **244**, 263–281.

KIRPEKAR, S.M., PRAT, J.C., PUIG, MARGARITA & WAKADE, A.R. (1972). Modification of the evoked release of noradrenaline from the perfused cat spleen by various ions and agents. *J. Physiol., Lond.*, **221**, 601–615.

KLEINHAUS, ANNA L. & PRICHARD, M.W. (1975). Calcium dependent action potentials produced by leech Retzius cells by tetraethylammonium chloride. *J. Physiol., Lond.*, **246**, 351–361.

KOKETSU, K. (1958). Action of tetraethylammonium chloride on neuromuscular transmission in frogs. *Am. J. Physiol.*, **193**, 213–218.

KÜLZ, F. (1922). Quantitative Untersuchungen über die wirksamkeit homologer quantärer Ammoniumbasen. *Pflügers Arch. ges. Physiol.*, **195**, 623–625.

MAANEN, E.F. VAN & WERTY, A.W. (1966). Vas deferens potentiation of response by tetraethylammonium. *Fedn Proc.*, **25**, 228.

SCHMIDT, H. & STÄMPFLI, R. (1966). Die Wirkung von Tetraäthylammonium-chloride auf den einzelnen Ranvierschen Schnürring. *Pflügers Arch. ges. Physiol.*, **287**, 311–325.

SCHÜLLER, J. (1920). Über physiologische und pharmakologische Versuche am Rektum des Frosches. *Arch. exp. Path. Pharmak.*, **90**, 196–241.

STJÄRNE, L. (1973). Michaelis-Menten kinetics of calcium dependence of sympathetic neurotransmitter secretion in guinea-pig vas deferens: Comparison between effects of phentolamine and of tetraethylammonium. *Acta physiol. scand.*, **89**, 142–144.

TASAKI, I. & HAGIWARA, S. (1957). Demonstration of two stable potential states in the squid giant axon under tetraethylammonium chloride. *J. gen. Physiol.*, **40**, 859–885.

THOENEN, H., HAEFELY, W. & STAEHELIN, H. (1967). Potentiation by tetraethylammonium of the response of the cat spleen to postganglionic sympathetic nerve stimulation. *J. Pharmac. exp. Ther.*, **157**, 532–540.

*(Received November 6, 1975.*
*Revised April 14, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 57–70

# THE KINETICS OF COMPETITIVE ANTAGONISTS ON GUINEA-PIG ILEUM

FIONA ROBERTS[1] & R.P. STEPHENSON
Department of Pharmacology, University of Edinburgh,
1 George Square, Edinburgh EH8 9JZ

**1** The kinetics of action of some competitive muscarinic and histamine antagonists were examined on guinea-pig isolated ileum and their behaviour compared with the predictions of the interaction-limited model described by Paton (1961).

**2** The kinetics of antagonism were not consistent with the predictions of this model: (1) The apparent dissociation rate constant calculated from the decrease in occupancy on washout was not independent of the concentration of antagonist. (2) The dissociation rate constant of a 'slow' antagonist calculated from the change in occupancy when a 'fast' antagonist was superimposed varied with the concentration of fast antagonist. (3) If the concentration of slow antagonist was increased when the fast antagonist was superimposed so that the equilibrium occupancy of the 'slow' was the same as before, a transitional phase was observed.

**3** The kinetics of antagonism were observed in longitudinal muscle strips and intact pieces of ileum, bathed in Tyrode or Krebs solution, and with isometric and isotonic recording. No evidence was found that the discrepancies between the interaction-limited model and the observed kinetics could be accounted for by the experimental method used.

**4** It is therefore concluded that either access is rate-limiting in these circumstances or, if interaction is rate-limiting, some alternative interaction-limited model is required to describe the kinetics of antagonism. In either case it would seem unwise at this time to calculate antagonist-receptor rate constants from the observed kinetics of antagonism.

## Introduction

If the mathematical model derived by Paton (1961) based on a simple mass action relationship describes how a competitive antagonist combines with its receptors, the affinity constant, $K$, of the antagonist will be the ratio of two constants, $k_1$ and $k_{-1}$, the rate constants describing the rate of association and dissociation from the receptors. Furthermore, if the kinetics of antagonism on an isolated tissue is limited by the rate at which the antagonist interacts with the receptors, values of $k_{-1}$, (and hence $k_1$ as $K = k_1/k_{-1}$), could be calculated from the observed kinetics of antagonism and differences in affinity between antagonists could be related to differences in $k_1$ and $k_{-1}$ separately (Paton, 1961; Paton & Rang, 1965).

Despite apparent agreement with this model in certain circumstances (Paton, 1961; Paton & Rang, 1965; Rang, 1966; Ginsborg & Stephenson, 1974) certain serious discrepancies have led to the suggestion that it is the rate of access to the receptors which governs the kinetic behaviour of antagonists (Thron & Waud, 1968). The following study was therefore undertaken to provide further evidence as to whether access to the receptor or interaction with the receptor normally limits the kinetics of competitive antagonists on guinea-pig ileum and hence whether it is reasonable to calculate rate constants from the observed kinetics. Part of this work has been communicated to the Pharmacological Society (Roberts & Stephenson, 1974).

## Methods

Intact pieces of ileum or longitudinal muscle strips were prepared from 150 to 400 g guinea-pigs as described by Edinburgh Staff (1968) and Paton & Rang (1965). The preparations were suspended in an organ bath containing a bathing solution at 36°C. Usually a Tyrode solution bubbled with air and of the following composition was used: (mM) $Na^+$ 149.2, $K^+$ 2.7, $Mg^{2+}$ 1.1, $Ca^{2+}$ 1.8, $Cl^-$ 143.2, $HCO_3^-$ 11.9, $H_2PO_4^{2-}$ 0.4, $SO_4^{2-}$ 1.1, glucose 5.6 and 27.6 hexa-

---

[1] Present address: Department of Pharmacology, University of Bristol, University Walk, Bristol BS8 1TD.

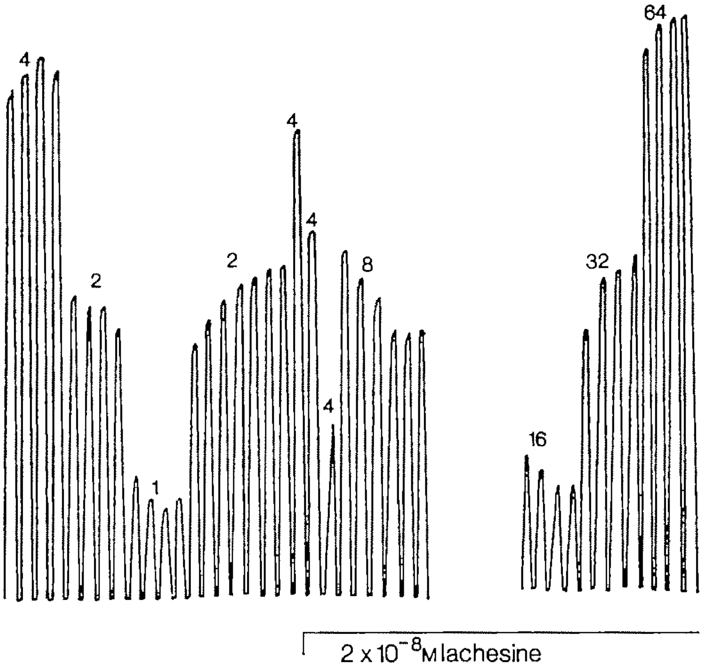


**Figure 1** The onset of action of lachesine illustrating the method used to follow the kinetics of antagonism. There is a gap in the trace of 28.5 minutes. The paper drive to the potentiometric recorder was on from just before the agonist was added to just after washout. The concentrations of agonist used are indicated above the responses — M × $10^7$, (i.e. 4 = 4 × $10^{-7}$ M).

methonium bromide. Alternatively a Krebs solution bubbled with 95% $O_2$ and 5% $CO_2$ was used: (mM) $Na^+$ 155.8, $K^+$ 5.6, $Mg^{2+}$ 1.1, $Ca^{2+}$ 2.2, $Cl^-$ 162.1, $HCO_3^-$ 1.79, glucose and $10^{-7}$ M atropine.

Events in the organ bath were controlled by automatic apparatus and the responses were recorded in an analogue and digital form (Stephenson, 1968; Abramson, Barlow, Mustafa & Stephenson, 1969). The fluid in the bath was changed at the appropriate time by upward displacement and overflow with the bathing solution, either alone or containing drug(s) at predetermined concentrations. The agonist was in contact with the tissue for 17 s before being washed out twice, with a 30 s interval between the washings. The agonist (either carbachol or histamine as appropriate) was applied every 90 seconds.

In most experiments the responses produced by the agonist were recorded with an isotonic lever with a differential transformer as a transducer. A weight of between 0.5 and 0.8 g was used to load the lever when intact pieces of ileum were used, and between 0.2 and 0.5 g when muscle strips were used. Alternatively, where specified, an isometric transducer was used (Devices Physiological Transducer 2.S.T.O.2).

The organ bath was connected via a two-way stopcock to either of two sets of 5 reservoirs, 4 containing agonist and one without. Turning the stopcock enabled a quick change to be made from a set of solutions not containing antagonist to a set all containing antagonist or *vice versa*. Similarly a change could be made from a set with one antagonist to a set with two.

Initially three concentrations of agonist were used as shown in Figure 1, the responses being within the 'linear' region of the log dose-response curve. When the tissue had settled down, usually in about 2–3 h, one concentration of agonist was repeated a number of times. The stopcock was then turned, usually during a contraction so that the peak of the following contraction occurred 90 s after the change. The establishment of the new equilibrium was followed by changing the concentrations of agonist applied so that the response size remained within a narrow range.

To follow the kinetics of onset and offset of antagonism the tissue was set up in the absence of antagonist and a log dose-response curve plotted from the last sequence of responses before the antagonist was added. A certain concentration of antagonist was then introduced and the dose-ratio, $DR_t$, corresponding to each response during onset determined from the curve and the corresponding antagonist occupancy $p_t$ calculated from the formula

$p_t = (DR_t - 1)/DR_t$. When equilibrium was established the antagonist was removed and offset followed in a similar way.

Occupancy changes during each experiment were plotted using the convention of Paton & Rang (1965). Values of $(p_\infty - p_t)$ or $p_t$ during onset or offset respectively were plotted on a log scale against time. If the relationship between log occupancy and time appeared to be reasonably linear time constants for the development and decline in occupancy were determined from the slopes of straight lines drawn through the points by eye. In those experiments where there did not appear to be a reasonably linear relationship, time constants corresponding to the initial rate were calculated from the first three responses (for lachesine or antazoline) or from the first response (for pentyl triethyl ammonium iodide (pentyl TEA)).

When studying the offset of benziloyltropine methyliodide (BTrMe) on superimposition of pentyl TEA, the tissue was initially equilibrated with a certain concentration of BTrMe. The fast antagonist was then superimposed and the change in dose-ratio followed. The offset was not followed to equilibrium in all experiments.

A log dose-response curve was plotted from the last sequence of responses before the changeover and dose-ratios corresponding to each subsequent response determined relative to this curve. BTrMe's occupancy corresponding to the response at time *t* after the changeover, $p_{S,t}$, was calculated from this dose-ratio in the following way: If $A_S$ and $A_{S+F,t}$ are the concentrations of agonist required to produce the same response in the presence of the slow antagonist alone and in the presence of slow and fast at time *t* after the changeover, the observed dose-ratio $DR_t$ will be equal to $A_{S+F,t}/A_S$. If $a$ is the concentration of agonist which would have been required to produce the same response in the absence of antagonists and $A_{S+F,t}/a$ is called $DR_{F+S,t}$ and $A_S/a$ is called $DR_S$: $DR_t = A_{S+F,t}/A_{SL} = DR_{F+S,t}/DR_S$. $DR_{F+S,t}$ is equal to the reciprocal of the proportion of free receptors, i.e. $DR_{F+S,t} = 1/(1 - p_{S,t} - p_F)$ and similarly $DR_{SL} = 1/(1 - p_S) = (1 + c_S K_S)$. Therefore the observed dose-ratio

$$DR_t = 1/[(1 - p_{S,t} - p_F)(1 + c_S K_S)].$$

If it is further assumed that the fast antagonist is at all times in equilibrium with the receptors not occupied by the slow, $p_F = c_F K_F(1 - p_{S,t})/(1 + c_F K_F)$. Therefore knowing $c_F$, $K_F$, $K_S$ and the observed dose-ratio $DR_t$, BTrMe's occupancy at time *t*, $p_{S,t}$ can be calculated.

The value of $K_F$ used was $3.6 \times 10^4\ \mathrm{M}^{-1}$. This corresponds to the mean of 24 estimations using concentrations of pentyl TEA between $0.3 \times 10^{-4}$ M and $8 \times 10^{-4}$ M (mean ± s.e. mean, $3.69 \pm 0.15 \times 10^4\ \mathrm{M}^{-1}$). This value agrees with that obtained by Abramson *et al.* (1968), (Log $K = 4.588$; $K = 3.87 \times 10^4\ \mathrm{M}^{-1}$). However, when higher concentrations of pentyl TEA were examined the mean dose-ratio was lower than expected. For instance the mean dose-ratio produced by $30 \times 10^{-4}$ M was 53.7 ($n = 6$) and not around 110 as expected. The reason for this change in apparent affinity is not clear as it did not appear to be associated with a change in slope of the log dose-response curve and was not more marked if agonists such as pentyl or hexyl trimethylammonium (both lower efficacy than carbachol) were used instead of carbachol. Pentyl TEA was originally selected as the fast antagonist in our experiments because it was the weakest readily available and so potentially the fastest; potent antagonists such as atropine with affinities around $10^9\ \mathrm{M}^{-1}$ and upwards are very slow whereas faster antagonists have lower affinities than this. However, the kinetics of some antagonists, such as desoxylachesine, with affinities in the $10^7$–$10^8\ \mathrm{M}^{-1}$ range, were subsequently found to act just as fast as the less potent pentyl TEA. It would therefore have been better to use a more potent antagonist than pentyl TEA, not only to avoid the anomalous dose-ratios produced by high concentrations but also to avoid the very large amounts of pentyl TEA used.

The value of $K_S$ used was $1.5 \times 10^{10}\ \mathrm{M}^{-1}$. This corresponds to the mean of 27 estimations using concentrations of BTrMe between $10 \times 10^{-10}$ and $200 \times 10^{-10}$ M (mean ± s.e. mean = $1.55 \pm 0.17 \times 10^{10}\ \mathrm{M}^{-1}$). This is somewhat lower than the value of $2.36 \times 10^{10}\ \mathrm{M}^{-1}$ obtained by Barlow & Mustafa (1968), but with these values of $K_S$ and $K_F$ the antagonists produced the expected combined dose-ratios even when concentrations of pentyl TEA greater than $8 \times 10^{-4}$ M were used.

For each experiment values of $(p_{S,t} - p_{S,\infty})$ were plotted on a log scale against time and as there was a reasonably linear relationship, time constants were determined from the slopes of the straight lines drawn through the points by eye.

As lachesine equilibrates so much more rapidly than BTrMe it was possible to follow first the onset of action of lachesine and then its offset on superimposition of pentyl TEA. Values of $p_{S,t}$ were calculated as above but using the equilibrium dose-ratios produced in that experiment by lachesine alone and in combination with pentyl TEA to estimate $(1 + c_S K_S)$ and $(1 + c_F K_F)$. The value of $(1 + c_F K_F)$ was consistent with the equilibrium dose-ratios produced by pentyl TEA alone even when high concentrations of pentyl TEA were used. This is therefore different, for some unknown reason, from the combination of pentyl TEA with BTrMe. Values of $(p_{S,t} - p_\infty)$ were plotted as before but $\tau_{\mathrm{off}}$ was calculated from the first three responses after the changeover since the relationship between log occupancy and time was not reasonably linear. The offset of lachesine on superimposition of desoxylachesine and octyl TMA was followed in a similar way.

The following drugs were kindly provided by Dr R.B. Barlow: benziloyltropine methyliodide (BTrMe); diphenylhydroxyacetoxyethyl dimethylethylammonium bromide (lachesine); diphenylacetoxyethyl dimethylethylammonium iodide (desoxylachesine); *n*-octyl trimethylammonium iodide (octyl TMA); *n*-pentyl triethyl ammonium iodide (pentyl TEA). Other drugs used were: antazoline hydrochloride (Ciba), atropine sulphate (BDH), carbaminoylcholine chloride (carbachol, BDH), hexamethonium bromide (Koch-Light), mepyramine maleate (May and Baker).

The following notation is used:

$c$, $c_S$, $c_F$ the concentration of a drug, of a slow antagonist and of a fast antagonist (M).

$p_o$, $p_t$, $p_\infty$ the proportion of receptors occupied by a drug initially, after a time $t$ and at equilibrium.

$p_S$, $p_F$ the proportion of receptors occupied by a slow antagonist and a fast antagonist.

$DR_t$, $DR_\infty$ the dose-ratio produced by an antagonist at time $t$, and at equilibrium.

$DR_{F+S}$, $DR_F$, $DR_S$ the combined dose-ratio produced by a fast and a slow antagonist acting together, by a fast alone, and a slow alone, all at equilibrium.

$K$, $K_S$, $K_F$ the affinity constant of an antagonist, of a slow antagonist and of a fast antagonist ($M^{-1}$).

$k_1$, $k_{-1}$ the antagonist receptor association and dissociation rate constants ($s^{-1}M^{-1}$, $s^{-1}$).

$\tau_{on}$, $\tau_{off}$ the time constant describing an increase or decrease in occupancy (s or min).

## I The kinetics of onset and offset of antagonism

Paton (1961) observed that, contrary to the predictions of the interaction-limited model, the rates of offset from high concentrations of atropine and mepyramine were not independent of the antagonist concentration and he suggested that this was due to appreciable intracellular uptake. These antagonists have therefore been re-examined together with quaternary compounds with which it was hoped to minimize this complication.

### *Theory*

If the rate of association and dissociation from the receptors determines the rate of increase or decrease of an antagonist's occupancy when it is added or removed from the bathing solution surrounding an isolated tissue then, (Paton, 1961), during onset:

$$p_t = p_\infty(1 - \exp[-(k_1c + k_{-1})t])$$

and during offset

$$p_t = p_o \exp[-k_{-1}t]$$

Therefore

1. The antagonist's occupancy should change exponentially during both the onset and offset of antagonism.
2. As the time constant for offset $\tau_{off}$ is equal to $1/k_{-1}$ it should be independent of concentration.
3. The ratio $\tau_{off}/\tau_{on}$ should be equal to the equilibrium dose-ratio.

If access were rate-limiting the predicted behaviour would depend on the particular access model being considered.

## Results (I)

As shown in Figure 2 and Table 1 the kinetics of antagonism do not appear to be consistent with the interaction model. In particular, following the onset from higher concentrations of BTrMe the rate of offset became faster and following higher concentrations of atropine, antazoline, lachesine, mepyramine and pentyl TEA the rate of offset became slower.

The increasing rate of recovery following exposure to higher concentrations of BTrMe is surprising as, at any time after about 10 min from the beginning of the washout period, the tissue is actually more sensitive to carbachol after having been treated with a high than a low concentration of BTrMe. As this was not observed when longitudinal muscle strips were used instead of intact pieces of ileum (Section IV, Table 5), the kinetics of BTrMe on intact pieces of ileum was reinvestigated subsequently and again the same observation was made.

These differences cannot be attributed to a change in affinity as significant differences in apparent affinity were not observed except with pentyl TEA (see Methods section). With pentyl TEA the rates of offset became appreciably slower at concentrations well below that at which this change in affinity was observed and so cannot be totally attributed to it.

In addition these discrepancies cannot be explained by intracellular uptake as they are not restricted to the non-quaternary compounds, as the change in rate of offset of BTrMe was not associated with a decrease in the time to which the tissue was exposed to the antagonist (about 3 h) and also increasing the time to which the tissue was exposed to $1 \times 10^{-4}$ M pentyl TEA from 9 to 30 min did not appear to influence the subsequent rate of offset.

Again, contrary to the predictions of the interaction limited model, some of the plots in Figure 2 do not appear to be linear but in view of the unavoidable changes in the sensitivity of the tissue with time, non-linearity of semilogarithmic plots for antagonists as slow as BTrMe is difficult to interpret. However, pentyl TEA and antazoline are so fast that the curvature of such offset plots can hardly be attributed to a change in sensitivity. The non-linearity of the onset plots with BTrMe and lachesine may also be an artifact as the time taken for equilibrium to be established was considerably less than that predicted from the

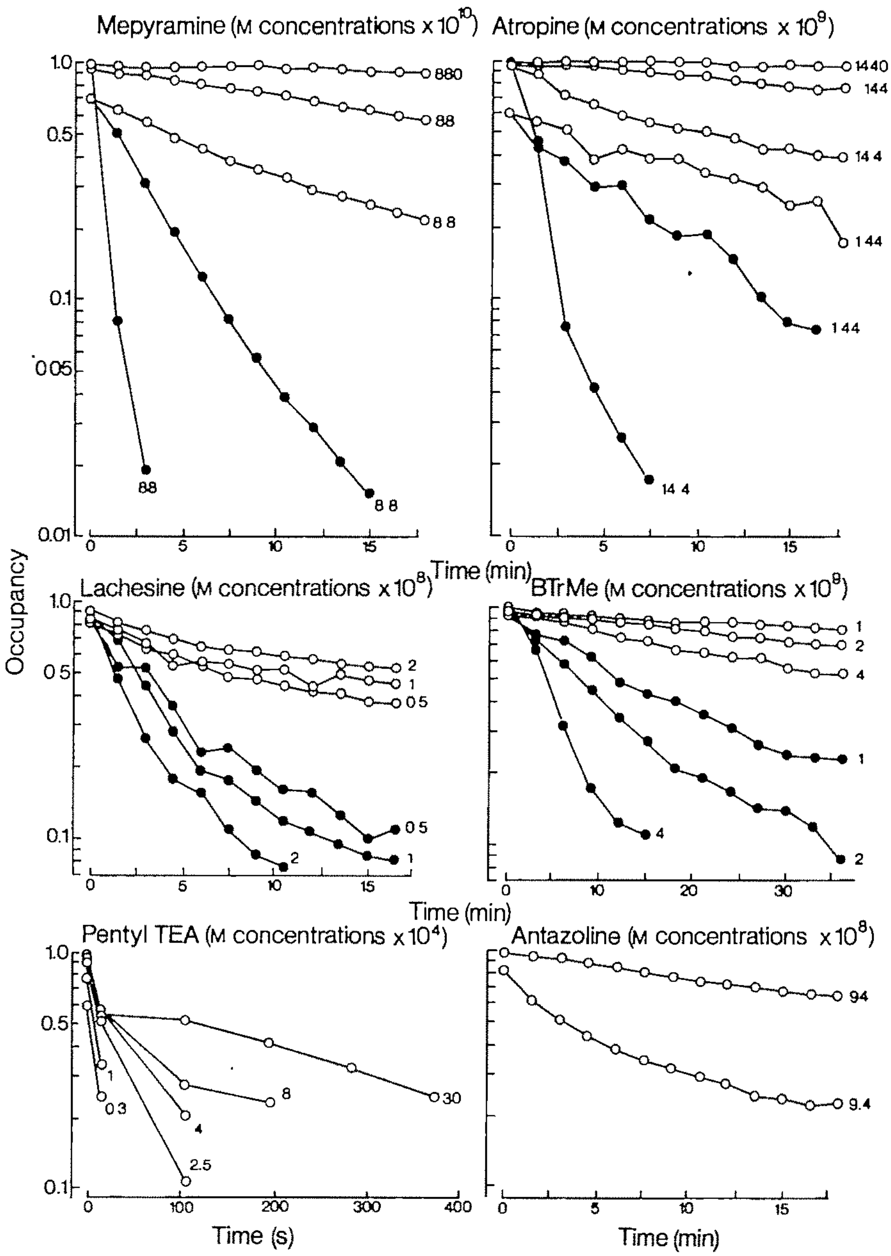


**Figure 2** The kinetics of onset and offset of six antagonists illustrating the relationship found between occupancy plotted on a log scale and time. The mean occupancy at the time corresponding to each response was calculated from the results of the individual experiments in each group (Table 2). These values are plotted using the convention of Paton & Rang (1965): for onset (closed circles) values of $(p_\infty - p_t)$, and for offset (open circles) values of $p_t$ were plotted on a log scale against time. If interaction were rate-limiting there would be a linear relationship between occupancy plotted on a log scale and time. Also $\tau_{off}$ would be constant for each antagonist. BTrMe = benziloyltropine methyliodide; Pentyl TEA = *n*-pentyl triethyl ammonium iodide.

occupancy plots. This may be due to the way in which the equilibrium occupancy was obtained. In these experiments, after an initial period during the onset using one concentration of agonist, three were then used until equilibrium appeared to have been established. This could be held to have produced, by desensitization or some such effect, a larger apparent equilibrium dose-ratio than would otherwise have been obtained and this would have induced curvature into the onset plots.

### II The 'displacement' of a slow antagonist on superimposition of a fast

Thron & Waud (1968) suggested that if access rather than interaction is rate-limiting the large concentration gradient resulting when a potent slow antagonist is 'displaced' from the receptors on superimposition of a fast antagonist might accelerate the removal of the slow antagonist from the tissue as compared to simply washing out the slow antagonist. This prediction has been investigated.

*Theory*

If interaction were rate-limiting and a fast antagonist superimposed on a tissue previously equilibrated with a very much slower antagonist, the fast antagonist can be considered to equilibrate instantaneously with the receptors not occupied by the slow. This would cause the 'displacement' of the slow antagonist according to the following equation (Rang, 1966):

$$p_{S,t} = p_{S,o} - (p_{S,\infty} - p_{S,o})$$
$$(\exp[-k_{-1}t(1 + c_S K_S + c_F K_F)/(1 + c_F K_F)]$$

and the fast antagonist would be at equilibrium at all time with the receptors not occupied by the slow.

If access were rate-limiting, although the predicted behaviour would depend on the particular model, the values of $k_{-1}$ obtained in this way might be expected to be larger than those obtained in I.

**Table 1** The kinetics of onset and offset of six antagonists. (Values are mean ± s.e. mean).

| n | *Concentration of antagonist (M)* | *Time constant for onset ($\tau_{on}$) (min)* | *Time constant for offset ($\tau_{off}$) (min) *(s)* | $\tau_{off}/\tau_{on}$ | *Equilibrium dose-ratio ($DR_\infty$)* |
|---|---|---|---|---|---|
| Atropine | | | | | |
| 1 | $1.44 \times 10^{-9}$ | 7.5 | 17 | 2.3 | 2.6 |
| 1 | $14.4 \times 10^{-9}$ | 1 | 20 | 20 | 21.4 |
| 3 | $144 \times 10^{-9}$ | | 45 ± 2 | | 355 ± 3.2 |
| 3 | $1440 \times 10^{-9}$ | | 437 ± 93 | | 3044 ± 62 |
| BTrMe | | | | | |
| 6 | $1 \times 10^{-9}$ | 27 ± 5 | 398 ± 82 | 15 ± 2 | 16 ± 1 |
| 5 | $2 \times 10^{-9}$ | 12 ± 2 | 120 ± 7 | 13 ± 2 | 36 ± 4 |
| 3 | $4 \times 10^{-9}$ | 6 ± 1 | 70 ± 5 | 12 ± 2 | 60 ± 2 |
| Lachesine | | | | | |
| 5 | $0.5 \times 10^{-8}$ | 6.7 ± 0.9 | 25.5 ± 2.7 | 4.0 ± 0.7 | 4.8 ± 0.4 |
| 7 | $1 \times 10^{-8}$ | 5.7 ± 0.4 | 22.3 ± 4.4 | 4.0 ± 0.7 | 7.4 ± 0.8 |
| 16 | $2 \times 10^{-8}$ | 3.7 ± 0.3 | 25.6 ± 3.0 | 7.6 ± 1.0 | 14.9 ± 1.0 |
| Pentyl TEA | | | | | |
| 4 | $0.3 \times 10^{-4}$ | | *17 ± 4 | | 2.8 ± 0.5 |
| 3 | $1 \times 10^{-4}$ | | *18 ± 2 | | 4.4 ± 0.2 |
| 4 | $2.5 \times 10^{-4}$ | | *33 ± 9 | | 11.1 ± 0.6 |
| 4 | $4 \times 10^{-4}$ | | *44 ± 12 | | 14.2 ± 0.4 |
| 5 | $8 \times 10^{-4}$ | | *36 ± 8 | | 27.9 ± 2.8 |
| 4 | $30 \times 10^{-4}$ | | *59 ± 17 | | 49.4 ± 5.2 |
| Antazoline | | | | | |
| 6 | $9.4 \times 10^{-8}$ | | 6.6 ± 0.4 | | 5.8 ± 0.4 |
| 6 | $94 \times 10^{-8}$ | | 42.5 ± 2.7 | | 40.7 ± 1.6 |
| Mepyramine | | | | | |
| 6 | $8.8 \times 10^{-10}$ | 3.5 ± 0.1 | 13.4 ± 0.8 | 4.0 ± 0.3 | 3.5 ± 0.1 |
| 10 | $88 \times 10^{-10}$ | 0.6 ± 0.01 | 35.9 ± 3.3 | 59.2 ± 6.4 | 26.5 ± 1.6 |
| 4 | $880 \times 10^{-10}$ | | 207 ± 28 | | 215 ± 19 |

BTrMe = benzilloyltropine methyliodide; pentyl TEA = *n*-pentyl triethyl ammonium iodide.

* If interaction were rate limiting, $\tau_{off}$ would be equal to $1/k_{-1}$ and so would be constant. Also $\tau_{off}/\tau_{on}$ would be equal to $DR_\infty$.

## Results (II)

Although the offset of BTrMe's occupancy on superimposition of pentyl TEA was reasonably exponential (Figure 3) the rate of offset was accelerated as compared with simply washing out BTrMe (Table 2). The degree of acceleration increased with the concentration of pentyl TEA, approaching a limiting value of about 20 min (Figure 4).

The offset of lachesine's occupancy on superimposition of pentyl TEA was not considered to be sufficiently exponential for time constants to be calculated from the mean slope of the semilogarithmic plots (Figure 5). Time constants corresponding to the initial rate were therefore calculated from the first three responses following the superimposition of pentyl TEA. These correspond to rates faster than would be predicted from the rate of recovery on removal of lachesine from the bathing solution (Figure 6 and Table 1). The limiting value of $k_{-1}$ for lachesine shown in Figure 6 is remarkably similar to that calculated by Paton & Rang (1965) from the rate of offset on removal of lachesine from the bathing solution. It is thus possible that some condition in Paton & Rang's experiments which we were unable to

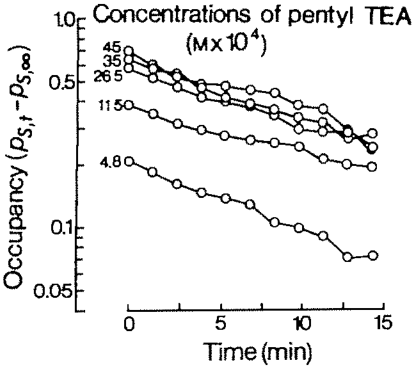


**Figure 3** The kinetics of offset of benziloyltropine methyliodide (BTrMe, $4\times10^{-9}$ M) when various concentrations of *n*-pentyl triethyl ammonium iodide (pentyl TEA) were superimposed. The mean occupancy of BTrMe at the times corresponding to each response ($\rho_{S,t}$) were calculated from the individual experiments in each group (Table 2). The values of ($\rho_{S,t}-\rho_{\infty}$) are plotted on a log scale against time. If BTrMe's interaction with the receptors were rate-limiting there would be a linear relationship between occupancy plotted on a log scale against time.

**Table 2** The kinetics of offset of benziloyltropine methyliodide (BTrMe) on superimposition of *n*-pentyl triethyl ammonium iodide (pentyl TEA). (Values are mean ± s.e. mean).

| n | *Concentration of BTrMe ($c_S$) (M)* | *Concentration of pentyl TEA ($c_F$) (M)* | $\tau_{off}\left[\frac{1+c_SK_S+c_FK_F}{1+c_FK_F}\right]$ *(min)* |
|---|---|---|---|
| 5 | $0.6\times10^{-9}$ | $2.2\times10^{-4}$ | 136.4 ± 23.4 |
| 4 | | $2.5\times10^{-4}$ | 100.0 ± 26.7 |
| 4 | | $5.4\times10^{-4}$ | 95.5 ± 17.2 |
| 4 | | $10.3\times10^{-4}$ | 63.0 ± 8.4 |
| 5 | | $25.0\times10^{-4}$ | 33.2 ± 6.7 |
| 4 | $1\times10^{-9}$ | $1.3\times10^{-4}$ | 148.5 ± 22.0 |
| 4 | | $2.5\times10^{-4}$ | 124.5 ± 21.3 |
| 5 | | $3.3\times10^{-4}$ | 132.4 ± 18.8 |
| 4 | | $6.0\times10^{-4}$ | 119.3 ± 16.3 |
| 4 | | $7.8\times10^{-4}$ | 85.3 ± 8.4 |
| 6 | | $14.0\times10^{-4}$ | 48.7 ± 5.8 |
| 4 | | $25.0\times10^{-4}$ | 30.5 ± 7.8 |
| 11 | $2\times10^{-9}$ | $2.5\times10^{-4}$ | 84.2 ± 8.4 |
| 9 | | $6.0\times10^{-4}$ | 113.7 ± 16.7 |
| 6 | | $14.0\times10^{-4}$ | 48.0 ± 5.5 |
| 8 | | $25.0\times10^{-4}$ | 43.3 ± 7.2 |
| 5 | $4\times10^{-9}$ | $4.8\times10^{-4}$ | 78.4 ± 19.7 |
| 5 | | $11.5\times10^{-4}$ | 53.0 ± 8.1 |
| 5 | | $26.5\times10^{-4}$ | 29.8 ± 4.5 |
| 4 | | $35.0\times10^{-4}$ | 23.8 ± 3.8 |
| 3 | | $45.0\times10^{-4}$ | 21.3 ± 0.9 |

If interaction were rate-limiting, $\tau_{off}(1+c_SK_S+c_FK_F)/(1+c_FK_F)$ would be equal to $1/k_{-1}$ and so would be constant.

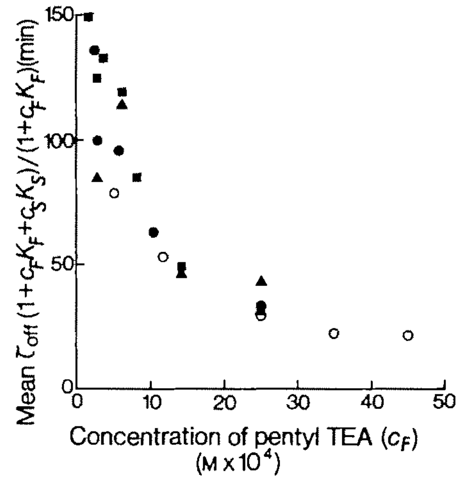


**Figure 4** The relationship between the rate of offset of benzilloyltropine methyliodide (BTrMe) on superimposition of *n*-pentyl triethyl ammonium iodide (pentyl TEA) and the concentration of pentyl TEA. Mean values of $\tau_{off}(1 + c_SK_S + c_FK_F)/(1 + c_FK_F)$ (Table 2) are plotted against $c_F$. The initial concentrations of BTrMe used, $c_S$ are indicated as follows: (●) $6 \times 10^{-10}$ M; (■) $10 \times 10^{-10}$ M; (▲) $20 \times 10^{-10}$ M; (O) $40 \times 10^{-10}$ M. If BTrMe's interaction with the receptors were rate-limiting $\tau_{off}(1 + c_FK_F + c_SK_S)/(1 + c_FK_F)$ would be equal to $1/k_{-1}$ and so would be constant and independent of $c_F$.

reproduce had the effect of greatly reducing the delay in drug access.

In view of the anomalous dose-ratios produced by high concentrations of pentyl TEA, the rates of offset of lachesine on superimposition of the different fast antagonists octyl TMA or desoxylachesine, were observed. These appeared to combine with lachesine as expected for two competitive antagonists:

$$DR_{F+S} = DR_F + DR_S - 1 = 1 + c_SK_S + c_FK_F.$$

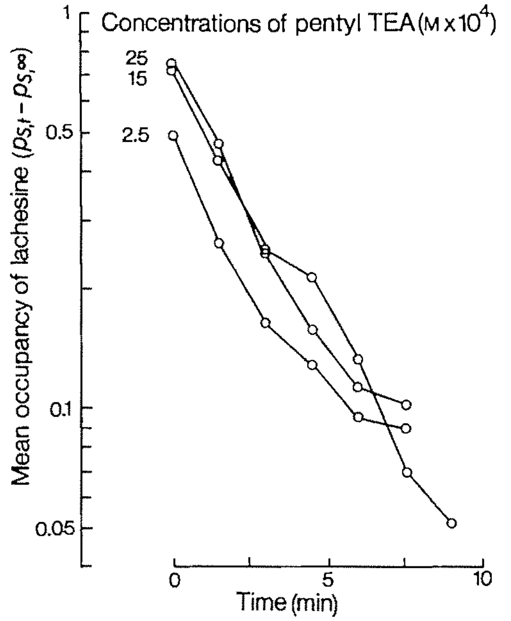


**Figure 5** The kinetics of offset of lachesine ($1 \times 10^{-8}$ M) when various concentrations of *n*-pentyl triethyl ammonium iodide (pentyl TEA) were superimposed. The mean occupancy of lachesine at the time corresponding to each response was calculated from the results of the individual experiments in each group (see Figure 6). Mean values of $(p_{S,t} - p_{S,\infty})$ are plotted on a log scale against time. If lachesine's interaction with the receptors were rate-limiting there would be a linear relationship between occupancy plotted on a log scale against time.

The rates observed (Table 3) are consistent with those obtained with pentyl TEA and so there is no reason to believe that the acceleration observed can be attributed to anomalous behaviour of pentyl TEA.

**Table 3** The rate of offset of lachesine ($2 \times 10^{-8}$ M) on superimposition of *n*-octyl trimethylammonium iodide (octyl TMA) or desoxylachesine

| n | *Fast antagonist* | *Concentration of fast antagonist ($c_F$)* *(M)* | $\tau_{off}\left[\frac{1 + c_SK_S + c_FK_F}{1 + c_FK_F}\right]$ *(min)* | *Observed dose-ratio* | *Calculated dose-ratio* $\left[\frac{1 + c_SK_S + c_FK_F}{1 + c_SK_S}\right]$ |
|---|---|---|---|---|---|
| 4 | Octyl TMA | $8.9 \times 10^{-4}$ | $8.1 \pm 2.7$ | $3.5 \pm 0.6$ | 4.9 |
| 4 | Octyl TMA | $15 \times 10^{-4}$ | $4.3 \pm 1.5$ | $7.8 \pm 1.0$ | 7.6 |
| 4 | Desoxylachesine | $.4 \times 10^{-4}$ | $2.6 \pm 0.5$ | $10.1 \pm 0.9$ | 11.9 |

It was assumed that for lachesine $K_S = 6.6 \times 10^8$ M$^{-1}$, (the mean value of $K \pm$ s.e. mean of 26 estimations was $6.6 \pm 0.4 \times 10^8$ M$^{-1}$), for octyl TMA $K_F = 6.3 \times 10^4$ M$^{-1}$ (Stephenson, 1956), and for desoxylachesine $K_F = 4 \times 10^7$ M$^{-1}$, (the mean value of $K \pm$ s.e. mean of 16 estimations was $4.0 \pm 0.3 \times 10^7$ M$^{-1}$).

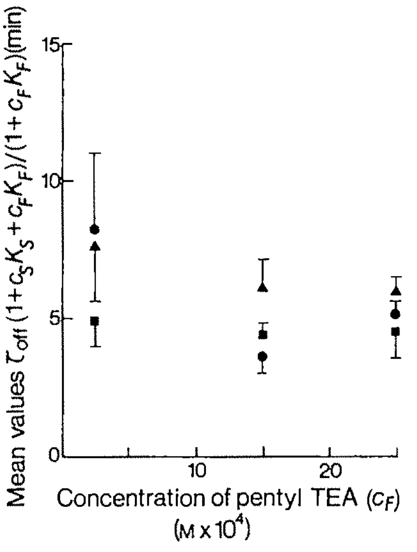


**Figure 6** The relationship between the rate of offset of lachesine on superimposition of various concentrations of *n*-pentyl triethyl ammonium iodide (pentyl TEA), and the concentration of pentyl TEA. Mean values are shown ($n=3$). Vertical lines show s.e. mean. The initial concentrations of lachesine, $c_S$ are indicated as follows: (●) $0.5\times10^{-8}$ M; (■) $1\times10^{-8}$ M; (▲) $2\times10^{-8}$ M. If interaction were rate-limiting'$\tau_{off}(1+c_S K_S+c_F K_F)/(1+c_F K_F)$ would be equal to $1/k_{-1}$ and so would be independent of $c_F$.

The calculations assume that the fast antagonist is so much faster than the slow that it can be considered to be in equilibrium at all times with the receptors not occupied by the slow. As the onset of all the concentrations of pentyl TEA examined were too fast to be measured this assumption is considered reasonable.

### III The interaction between BTrMe and pentyl TEA

The interaction between BTrMe and pentyl TEA was also followed with a slightly different procedure from that in II. As before the tissue was initially equilibrated with a concentration $c_{S'}$ of BTrMe but when the concentration, $c_F$, of pentyl TEA was superimposed the concentration of BTrMe was increased to $c_{S''}$, $c_{S''}$ being equal to $c_{S'}(1+c_F K_F)$, $K_F=3.6\times10^4$ M$^{-1}$. In this way the occupancy of BTrMe in equilibrium with the fast should be the same as before, although the total occupancy is increased.

Therefore if interaction were rate-limiting, although the fast antagonist would equilibrate very quickly with the receptors not occupied by the slow, the increased concentration of BTrMe would prevent it being 'displaced'. As BTrMe's occupancy is unaltered the degree of antagonism would change from one level to the next with no intermediate transitional stage.

If access were rate-limiting the concentration of fast antagonist in the proximity of the receptors would rise quickly to its equilibrium level and so the fast would equilibrate quickly with the receptors not occupied by

**Table 4** The interaction between benzilloyltropine methyliodide (BTrMe) and *n*-pentyl triethyl ammonium iodide (pentyl TEA)

| n | *Initial concentration of BTrMe* ($c_{S'}$) *(M)* | *Concentration of superimposed pentyl TEA* ($c_F$) *(M)* | **Size of transitional stage* | *Observed dose-ratio (±s.e. mean)* | *Calculated dose-ratio* $\left[\frac{1+c_{S''}K_S+c_F K_F}{1+c_{S'}K_S}\right]=(1+c_F K_F)$ |
|---|---|---|---|---|---|
| 1 | $2\times10^{-9}$ | $1\times10^{-4}$ | 1.1 | 4.7 | 4.6 |
| 1 | $2\times10^{-9}$ | $4\times10^{-4}$ | 1.2 | 17.9 ± 5.1 | 15.4 |
| 1 | $4\times10^{-9}$ | | 1.4 | | |
| 2 | $1\times10^{-9}$ | $8\times10^{-4}$ | 1.5, 1.4 | 39.4 ± 3.2 | 29.8 |
| 1 | $2\times10^{-9}$ | | 2.5 | | |
| 2 | $4\times10^{-9}$ | | 2.6, 2.8 | | |
| 3 | $1\times10^{-9}$ | $16\times10^{-4}$ | 1.7, 3.1, 2.0 | 55.3 ± 87.3 | 58.6 |
| 2 | $2\times10^{-9}$ | | 2.2, 2.1 | | |
| 2 | $4\times10^{-9}$ | | 3.1, 1.7 | | |

* The ratio of the equilibrium dose-ratio to the dose-ratio corresponding to the first response after the changeover gives an estimate of the size of the transitional stage. If interaction were rate-limiting the degree of antagonism would change from one level to the next with no transitional stage (i.e. 'size' = 1).

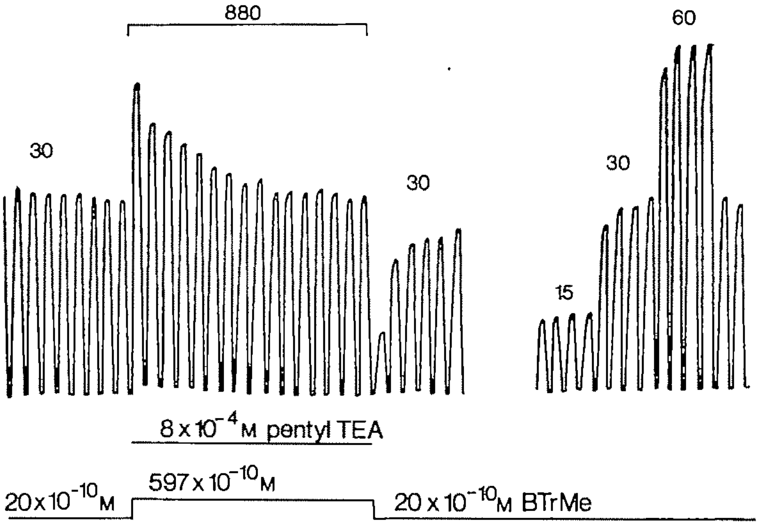


**Figure 7** The interaction between benzilloyltropine methyliodide (BTrMe) and *n*-pentyl triethyl ammonium iodide (pentyl TEA), the concentration of BTrMe being adjusted as shown. There is a gap in the trace of 57 minutes. The paper drive to the potentiometric recorder was on from just before the agonist was added to just after washout. The concentrations of agonist used are indicated above the responses — M x 10$^7$. If BTrMe's interaction with the receptors were rate-limiting, on superimposing pentyl TEA the degree of antagonism would change from one level to the next with no transitional stage.

the slow antagonist. The concentration of slow in the proximity of the receptors would, however, only rise slowly to its new level and so, because of this lag, the fast antagonist would initially 'displace' the slow antagonist from the receptors, the slow antagonist's occupancy being subsequently restored as its concentration in the proximity of the receptors rises to its new level.

## Results (III)

When the concentration of fast antagonist was sufficiently large a transitional stage was observed (Figure 7, Table 4). This stage is consistent with that expected if access were rate-limiting. The extent of the initial displacement increased with the concentration of pentyl TEA as would be expected from Figure 4. Subsequently BTrMe's occupancy was slowly restored to a level consistent with that calculated taking $K_F$ as $3.6 \times 10^4$ M$^{-1}$. In view of the very fast rate of onset of pentyl TEA it is unlikely that the transitional stage reflects the kinetics of this antagonist. Also the dose-response curve appeared to have shifted in a parallel manner following the superimposition of the fast antagonist and so there is no reason to believe that the agonist exposure time was insufficient for an equilibrium to be established.

## IV The effect of experimental method on the kinetics of antagonism

The results of kinetic studies by different groups of workers show large differences. For instance Paton (1961), using intact pieces of ileum, found that on washout atropine's occupancy declined with a half time of about 40 min whereas Paton & Rang (1965), using muscle strips, observed half times of about 7 min although the equilibrium constants were about the same. It was therefore suggested that the observed kinetics varied with the preparation used. In addition the studies of Beraldo & Rocha e Silva (1949) and of Paton & Rothschild (1965) suggest that the kinetics of antagonism are influenced by the composition of the bathing fluid. The effect of experimental method on the kinetics of antagonism was therefore investigated in order to determine whether the discrepancies between the observed kinetics and the interaction-limited model were associated with the method used.

## Results (IV)

The kinetics of BTrMe was examined using muscle strips and an isometric transducer instead of intact pieces of ileum and an isotonic lever. Both onset and offset (Table 5) were slower than those obtained with intact pieces of ileum (Table 1), and the rate of offset did not appear to depend on the concentration. Nevertheless interaction cannot be rate-limiting as the values of $\tau_{off}$ are again considerably larger than predicted from the rate of offset on superimposition of high concentrations of pentyl TEA.

Since BTrMe leaves the receptors slowly the agonist may fail to achieve equilibrium with the receptors. Such a failure would produce a non-parallel shift in the log dose-response curve with a depression of the maximum response. Also the speed of contraction is sometimes reduced. Experiments were performed to determine the minimum concentration of BTrMe at which such effects occurred. With the isotonically loaded intact ileum this was found to be about $2 \times 10^{-8}$ M which is comfortably above the concentration used in the experiments recorded in Table 1. With isometric muscle strips the effects occurred with much lower concentrations of BTrMe, down to $5 \times 10^{-9}$ M, just above the concentrations used in the experiments summarized in Table 5. However, in the experiments of Table 5 there was no indication of failure of the agonist to achieve equilibrium and so it seems unlikely that such an effect could be responsible for the difference between these results and those of Table 1. The difference might be a consequence of varying degrees of damage during the preparation of the tissue perhaps causing changes in the ionic content of the tissue.

The kinetics of lachesine was also investigated under different conditions. In each experiment two pieces of ileum from the same animal were used to follow the onset and offset of action of $2 \times 10^{-8}$ M lachesine and its rate of offset on superimposition of $15 \times 10^{-4}$ M pentyl TEA. The four combinations of lever (isotonic or isometric) and preparation (intact pieces of ileum or muscle strips) were investigated first in Tyrode solution, the order of the experiments being determined randomly. Subsequently the kinetics of lachesine were investigated in Krebs solution. In none of these experiments (Table 6) was the kinetics of onset and offset as fast as those predicted from the rate of offset on superimposition of a fast antagonist. It is therefore unlikely that the discrepancies between the observed kinetics and the interaction-limited model are associated with the bathing medium, preparation or recording method used. Also the discrepancy

**Table 5** The rates of onset and offset of benziloyltropine methyliodide (BTrMe) in muscle strips with the responses recorded isometrically. (Values are mean ± s.e. mean).

| n | *Concentration of BTrMe ($c_S$) (M)* | *Time constant for onset ($\tau_{on}$) (min)* | *Time constant for offset ($\tau_{off}$) (min)* | $\tau_{off}/\tau_{on}$ | *Equilibrium dose-ratio ($DR_\infty$)* |
|---|---|---|---|---|---|
| 3 | $1 \times 10^{-9}$ | 33 ± 12 | 578 ± 177 | 21 ± 8 | 25 ± 8 |
| 6 | $2 \times 10^{-9}$ | 20 ± 3 | 509 ± 60 | 27 ± 5 | 38 ± 5 |
| 4 | $4 \times 10^{-9}$ | 13 ± 4 | 502 ± 69 | 47 ± 9 | 77 ± 15 |

**Table 6** The kinetics of lachesine ($2 \times 10^{-8}$ M) using 6 combinations of preparation (Intact pieces of ileum or muscle strips), bathing medium (Krebs or Tyrode) and lever (isotonic or isometric). (Values are mean ± s.e. mean).

| n | *Ileum prepn* | *Bathing medium* | *Lever* | $\tau_{on}$ *(min)* | $^1\tau_{off}$ *(min)* | $^2\tau_{off}\dfrac{1 + c_SK_S + c_FK_F}{1 + c_SK_S}$ *(min)* | $DR_S$ |
|---|---|---|---|---|---|---|---|
| 4 | Intact | Tyrode | Isometric | 4.0 ± 0.6 | 40.4 ± 9.7 | 7.8 ± 1.1 | 15.7 ± 0.7 |
| 4 | Strips | Tyrode | Isometric | 6.6 ± 2.8 | 36.8 ± 12.9 | 7.3 ± 2.6 | 15.0 ± 1.0 |
| 4 | Intact | Tyrode | Isotonic | 4.0 ± 0.3 | 32.4 ± 7.8 | 5.6 ± 1.1 | 15.6 ± 1.9 |
| 4 | Strips | Tyrode | Isotonic | 7.6 ± 1.9 | 33.9 ± 6.4 | 4.3 ± 1.0 | 17.8 ± 0.9 |
| 4 | Intact | Krebs | Isotonic | 3.7 ± 0.3 | 18.5 ± 4.5 | 5.9 ± 1.0 | 12.6 ± 1.0 |
| 4 | Strips | Krebs | Isotonic | 2.8 ± 0.3 | 23.0 ± 3.4 | 4.7 ± 0.3 | 15.5 ± 0.6 |

$^1\tau_{off}$ was calculated from the rate of offset of lachesine on washout; $^2\tau_{off}$ was calculated from the rate of offset on superimposition of pentyl TEA. If interaction were rate-limiting, $^1\tau_{off}$ and $^2\tau_{off}(1 + c_SK_S + c_FK_F)/(1 + c_FK_F)$ would both be equal to $1/k_{-1}$ and so would themselves be equal.

between the kinetics of atropine when measured by Paton (1961) and when measured by Paton & Rang (1965) cannot be due to the former using intact pieces of ileum and the latter using muscle strips. Other workers have not found the kinetics of atropine to be faster when muscle strips are used: Furchgott (quoted by Paton, 1967b) using intact pieces of ileum measured offset half times nearer 10 min than 40 whereas Thron & Waud (1968, Figure 8) using muscle strips observed rates of offset considerably slower than 10 minutes.

## Discussion

In these experiments the kinetics of onset and offset of competitive antagonists do not appear to be compatible with the simple mass action model of Paton (1961). This is consistent with kinetic measurements on many other tissues (Furchgott, 1955; Paton, 1967a; Waud, 1967; Thron & Waud, 1968). Therefore either access of the antagonist to the receptors is rate-limiting or, if interaction with the receptors is rate-limiting, some alternative model is required to describe the kinetics of antagonism. If access is rate-limiting, the rate of offset of a slow antagonist on superimposing a sufficiently high concentration of a fast antagonist may allow the determination of $k_{-1}$ and if this is so the results of Figure 4 indicate that BTrMe dissociates slowly from the muscarinic receptors with a time constant of about 20 minutes. This would largely account for its high potency which in turn would account for the extremely slow onset of its action. Unfortunately without a clear idea of the nature of the access limitation the possibility that some other access limitation is operating when high doses of the fast antagonist are superimposed cannot be ruled out. Similarly if interaction with the receptors is rate-limiting, it would not seem reasonable to calculate antagonist receptor rate constants from the observed kinetics without a plausible new interaction model. The problem is therefore can an interaction or access-limited model be derived which adequately describes the observed kinetics?

The variability of kinetic measurements would seem more probable in an access-limited than in an interaction-limited situation. Not only do the kinetic measurements of a single group show considerable variation as was noted by Thron & Waud (1968) and also in this study, but also there are discrepancies between the observations of different groups of workers. The behaviour of atropine has already been mentioned. Similarly in this study lachesine's occupancy declined on washout with a time constant greater than 20 min whereas Paton & Rang (1965) observed rates corresponding to time constants of about 4 minutes.

If then the kinetics of antagonism reflect the rate at which the concentration of drug in the proximity of the receptors approaches that in the bathing medium, what determines this rate? For potent slow antagonists such as atropine and BTrMe this cannot be limited by diffusion alone as the difference between their speed and that of a weak antagonist such as pentyl TEA cannot be explained in terms of their molecular weight. Also, using the method of analysis described by Thron (1972), the rates of change of dose-ratio were not found to be consistent with simple diffusion or any other situation describable by a linear multicompartmental model.

If, however, the amount of drug taken up by the receptors is not quantitatively negligible, as the binding studies of Paton & Rang (1965) suggest, this would introduce non-linearity into the system and would also explain why only potent antagonists exhibit very slow onset and offset of action. Because of their potency such antagonists are tested in low concentrations and so the amount of drug taken up by the receptors will be appreciable compared with the amount in the volume with which the receptors equilibrate. This will delay the attainment of an equilibrium in the proximity of the receptors and so will slow the onset and offset of antagonism. With a weak antagonist a much higher concentration has to be used to produce the same degree of receptor block. Therefore the amount of drug taken up is small compared with that in the proximity of the receptors and so uptake will have a negligible effect on the approach to equilibrium. The apparent acceleration seen in Figure 4 can also be explained in this way.

Thron & Waud (1968) used a very similar argument to explain their observation that pretreatment with an irreversible antagonist increased the rate of onset and offset of a slow antagonist. They considered that the kinetics of antagonism may reflect the rate at which the antagonist penetrates through the extracellular space by diffusion, this being slowed by uptake onto the receptors on the more peripheral cells. Alternatively though, the above effects might be due to there being some sort of barrier between the bulk of the extracellular space and the receptors. The antagonist might then equilibrate relatively quickly with the bulk of the extracellular space and the kinetics of antagonism reflect the rate at which the concentration of drug to which the receptors are exposed approaches that in the bulk of the extracellular space, this being slowed by uptake onto the receptors.

The possibility of a barrier was discarded by Rang (1966) because he felt that, for guinea-pig isolated ileum, there was no physical structure which could act as such a barrier. However, electron micrographs show that between the trilaminar membrane and the bulk of the extracellular space is a layer called the basement membrane. Greengard & Straub (1958) suggested that this layer might be responsible for the apparently slow rates of diffusion of ions that they observed. If the diffusion of ions is affected by this layer might not the diffusion of drugs be also? Colquhoun & Ritchie (1972), in fact, mentioned the possibility that the kinetics of tetrodotoxin might be affected by this layer.

A barrier would more readily explain the effect of ionic environment on the kinetics of antagonists seen here and by Paton & Rothschild (1965) than a change in the rate of penetration. It would also explain why the kinetics are not faster when strips are used rather than intact pieces of ileum, although this could alternatively be due to the folding of the strips in the organ bath or to the agonist acting by setting up a pacemaker as was considered by Thron & Waud (1968). This would also predict that the kinetics of binding to tissue homogenates might not be appreciably faster than the kinetics of antagonism even if the kinetics of antagonism were access-limited. However, the barrier would have to have rather extraordinary powers of discrimination if it were to explain the transition stage seen in Figure 7 and the finding that in certain conditions the offset of BTrMe is faster following equilibration with higher concentrations. If the basement membrane is considered to be the most likely candidate for this barrier it may be possible to derive a plausible new kinetic model when more information is available concerning if and how such a membrane behaves in this way.

Alternatively if interaction is rate-limiting, a pacemaker mechanism of agonist action might seem to be necessary to explain why uptake onto the receptors does not cause appreciable slowing of antagonisms. If estimates of the binding capacity (M) of the muscarinic receptors are considered together with estimates of the size of the extracellular space (largest estimate of V), for slow antagonists the value of MK/V (Colquhoun & Ritchie, 1972) would seem to be sufficiently large (> 400) for appreciable slowing to be expected. However, if agonists act by setting up a pacemaker in the more peripheral cells, the kinetics of antagonism would reflect equilibration of the antagonist with these cells and not the rate of penetration through the tissue however slow this might be. This would predict, as Thron & Waud (1968) pointed out, a difference between the kinetics of antagonism and the kinetics of binding to intact tissues as seen by Paton & Rang (1965).

Before a new interaction model can be derived, modification of the simple mass action model would seem to be necessary to accommodate the change in the apparent affinity of pentyl TEA with concentration and also its combined dose-ratios with BTrMe and lachesine (see Methods section). Another anomalous observation was also made; in 39 out of 40 experiments the dose-response curve in the presence of desoxylachesine was slightly steeper than in its absence, this increase being too small to affect appreciably the calculation of dose-ratio. Both these observations could be attributed to the receptor existing in different forms.

In addition a plausible new interaction model would have to explain how the kinetics of antagonism can change without changing the apparent affinity at equilibrium. For instance pretreatment with an irreversible antagonist (Thron & Waud, 1968) and superimposing a high concentration of a fast antagonist, accelerate the kinetics of offset of a slow antagonist without altering the apparent equilibrium position. Similarly the variation in kinetic measurements already discussed does not seem to be associated with changes in the apparent affinity at equilibrium. There is also some evidence that ion levels can influence the observed kinetics of antagonism without changing the apparent affinity at equilibrium (Beraldo & Rocha e Silva, 1949; Paton & Rothschild, 1965), but in this study, although the bathing medium does appear to influence slightly the observed kinetics of lachesine (Table 6), the equilibrium dose-ratios were also slightly affected ($0.01 < P < 0.05$: 2 factor analysis of variance — interaction not significant). Differences in rates in these experiments may therefore be partly associated with changes in the apparent affinity constant.

In conclusion, it may be possible to derive a kinetic model based on a modification of the simple mass action model which is reasonably consistent with the observed kinetics and is also consistent with the available information concerning the binding of muscarinic agonists and antagonists to the receptors. However, without such a model or a plausible access model it would seem impossible at this time to decide whether access or interaction normally limits the kinetics of potent competitive antagonists on isolated pieces of guinea-pig ileum, or even whether both factors contribute to the observed kinetics of antagonism.

FR is grateful to the MRC for a training award.

## References

ABRAMSON, F.B., BARLOW, R.B., MUSTAFA, M.G. & STEPHENSON, R.P. (1969). Relationships between chemical structure and affinity for acetylcholine receptors. *Br. J. Pharmac.*, **37**, 207–233.

BARLOW, R.B. & MUSTAFA, M.G. (1968). Some derivatives of tropine and pseudotropine. *Br. J. Pharmac. Chemother.*, **34**, 389P.

BERALDO, W.T. & ROCHA e SILVA, M. (1949). Biological assay of antihistamines, atropine and antispasmodics upon the guinea-pig gut. *J. Pharmac. exp. Ther.*, **97**, 388–398.

COLQUHOUN, D. & RITCHIE, J.M. (1972). The kinetics of the interaction between tetrodotoxin and mammalian nonmyelinated nerve fibres. *Mol. Pharmac.*, **8**, 285–292.

EDINBURGH STAFF (1968). *Pharmacological Experiments on Isolated Preparations* p 58. Edinburgh & London: E. & S. Livingstone.

FURCHGOTT, R.F. (1955). The pharmacology of vascular smooth muscle. *Pharmac. Revs*, **7**, 183–265.

GINSBORG, B.L. & STEPHENSON, R.P. (1974). On the simultaneous action of two competitive antagonists. *Br. J. Pharmac.*, **51**, 287–300.

GREENGARD, P. & STRAUB, R.W. (1958). After-potentials in mammalian non-myelinated nerve fibres. *J. Physiol., Lond.*, **144**, 442–464.

PATON, W.D.M. (1961). A theory of drug action based on the rate of drug-receptor combination. *Proc. Roy. Soc. B*, **154**, 21–69.

PATON, W.D.M. (1967a). Adrenergic receptors viewed in light of general receptor theories. *Ann. N.Y. Acad. Sci.*, **139**, 632–641.

PATON, W.D.M. (1967b). Kinetic theories of drug action with special reference to the acetylcholine group of agonists and antagonists. *Ann. N.Y. Acad. Sci.*, **144**, 869–881.

PATON, W.D.M. & RANG, H.P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. Roy. Soc. B*, **163**, 1–44.

PATON, W.D.M. & ROTHSCHILD, A.M. (1965). The effect of varying calcium concentration on the kinetic constants of hyoscine and mepyramine antagonism. *Br. J. Pharmac. Chemother.*, **24**, 432–436.

RANG, H.P. (1966). The kinetics of action of acetylcholine antagonists in smooth muscle. *Proc. Roy. Soc. B*, **164**, 488–510.

ROBERTS, F. & STEPHENSON, R.P. (1974). The kinetics of action of acetylcholine antagonists on guinea-pig ileum. *Br. J. Pharmac.*, **51**, 128P.

STEPHENSON, R.P. (1956). A modification of receptor theory. *Br. J. Pharmac. Chemother.*, **11**, 379–393.

STEPHENSON, R.P. (1968). Automatic control of isolated smooth muscle with digital print-out of contraction size. *Br. J. Pharmac.*, **34**, 230P.

THRON, C.D. (1972). Non-linear kinetics of atropine acting on the pacemaker of the isolated guinea-pig atrium. *J. Pharmac. exp. Ther.*, **160**, 91–105.

THRON, C.D. & WAUD, D.R. (1968). The rate of action of atropine. *J. Pharmac. exp. Ther.*, **160**, 91–105.

WAUD, D.R. (1967). The rate of action of competitive neuromuscular blocking agents. *J. Pharmac. exp. Ther.*, **158**, 99–114.

*(Received December 8, 1975.
Revised March 16, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 71–79

# THE CAT LUNG STRIP AS AN *in vitro* PREPARATION OF PERIPHERAL AIRWAYS: A COMPARISON OF $\beta$-ADRENOCEPTOR AGONISTS, AUTACOIDS AND ANAPHYLACTIC CHALLENGE ON THE LUNG STRIP AND TRACHEA

K.M. LULICH, H.W. MITCHELL & M.P. SPARROW
Department of Pharmacology, The University of Western Australia, The Medical School,
Perth Medical Centre, Shenton Park, Western Australia 6009

1 A new *in vitro* preparation, the isolated lung strip of the cat, is described for investigating the direct effect of drugs on the smooth muscle of the peripheral airways of the lung. The preparation comprises a thin strip of lung parenchyma which can be mounted in a conventional organ bath for isometric tension recording. Its pharmacological responses have been characterized and compared with the isolated tracheal preparation of the cat.

2 The lung strip exhibited an intrinsic tone which was relaxed by catecholamines, aminophylline and flufenamate. It was contracted strongly by histamine, prostaglandin $F_{2\alpha}$, acetylcholine, compound 48/80, potassium depolarizing solution and alternating current field stimulation. In contrast, the cat trachea was unresponsive to histamine and prostaglandin $F_{2\alpha}$ and did not exhibit an intrinsic tone.

3 (–)-Isoprenaline and (–)-adrenaline were much more potent in relaxing the lung strip than the trachea. The potency order of relaxation responses to isoprenaline, adrenaline and (±)-noradrenaline in the lung strip was isoprenaline > adrenaline > noradrenaline but in the trachea was isoprenaline > noradrenaline $\geqslant$ adrenaline.

4 $\beta_2$-Adrenoceptor selective agonists salbutamol and terbutaline were more potent in the lung strip than the trachea, suggesting $\beta_2$-adrenoceptors predominated in the lung strip. Propranolol was equipotent in inhibiting isoprenaline relexations of the lung strip and trachea, whereas practolol was much less effective in inhibiting lung strip than trachea, further supporting a predominance of $\beta_2$-adrenoceptors in lung strip and $\beta_1$-adrenoceptors in trachea.

5 Strong Schultz-Dale type contractions were elicited in both lung strips and trachea by *Ascaris lumbricoides* antigen in actively sensitized cats. The initial phase of the contractile response of the lung strip following challenge was shown to be due to histamine release and was absent in the trachea. The delayed phase of the contraction which took several minutes to develop in both the mepyramine-treated lung strip and trachea was not due to prostaglandins $E_1$, $F_{2\alpha}$ or bradykinin, the probable mediator being slow reacting substance of anaphylaxis (SRS-A).

6 It is concluded that the isolated lung strip of the cat is useful as an *in vitro* model for investigating the effect of drugs on the smooth muscle of the peripheral airways of the lungs.

## Introduction

Techniques available for investigating the direct actions of drugs on smooth muscle of the lung chiefly use preparations of the large central airways. Tracheal rings tied together (Castillo & De Beer, 1947) or opened before tying together (Akcasu, 1959) and intraluminal pressure recorded from whole trachea (Farmer & Coleman, 1970) are examples. However, *in vivo* measurements of lung compliance or airways resistance suggest that the principal site of action of some drugs is at the level of the fine peripheral airways (Colebatch, 1970; Drazen & Austen, 1974). Direct evidence for such an action requires an *in vitro* preparation of the peripheral airways and we describe here a simple technique using strips of isolated lung parenchyma of the cat for investigating the direct effect of drugs on the small airways. Some of the pharmacological characteristics of this cat isolated lung preparation are described. A preliminary report of this work was presented at the 6th International Congress of Pharmacology (Sparrow & Mitchell, 1975).

## Methods

Adult cats of either sex were lightly anaesthetized with halothane, the chest opened, the lungs carefully excised and placed in modified Krebs solution at 37°C and aerated with 95% $O_2$ and 5% $CO_2$. The composition was (mM): NaCl 119, KCl 5.6, $NaHCO_3$ 14, $CaCl_2$ 2, dextrose 11 and $KH_2PO_4$ 1.2. The solution was buffered to pH 7.3 with NaMOPS (morpholinopropane-sulphonic acid titrated with NaOH to pH 7.3) 5 mM; $K_2H_2EGTA$ (ethyleneglycol-bis-($\beta$-amino-ethylene ether) *N,N'*-tetra-acetic acid) 0.05 mM was included as a sequestering agent as a safeguard against heavy metal contamination. In potassium depolarizing solution the NaCl in the above solution was replaced by $K_2SO_4$ 80 mM.

### *Preparation of isolated trachea from cats*

From tracheal rings about 3 mm in width, most of the cartilage was removed before attaching threads to the small pieces of cartilage remaining at each end of the trachealis muscle. The tissues were stretched to a tension of 0.3 g and where appropriate, tone was induced with 0.4 μM carbachol.

### *Preparation of isolated lung strips of cats*

Sections of lung tissue, about 3 mm in width, were dissected quickly from a lower lobe with the longitudinal axis of the strip cut parallel to the bronchus. This was then divided into two or more thin strips with approximate dimensions of 20 x 3 x 3 mm. (Figure 1).

In some experiments, strips were dissected from either the peripheral margin of a lobe or from across the lobe at right angles to the bronchus, but not including it. There was no detectable difference in the pharmacological responses of these strips compared with those routinely cut as in Figure 1.

With threads attached to each end, strips were mounted in organ baths containing aerated Krebs at 37°C. A load of between 2.0 to 2.5 g force was applied by gently stretching and the tissues were allowed to equilibrate for 1 h with the solution changed at 15 min intervals.

Usually two-organ baths, each containing a pair of strips, were run simultaneously to permit the testing of various drugs in differing order and dose and with adequate controls. Consistent responses could be obtained at 37°C for at least 6–8 h and the responsiveness to drugs was unimpaired after storage overnight at 4°C. Concentration-response relationships were obtained using a cumulative dose schedule after which the organ bath was flushed and the tension allowed to return to its original value. Changes in tension were measured isometrically with a Grass FTO3C force-displacement transducers.

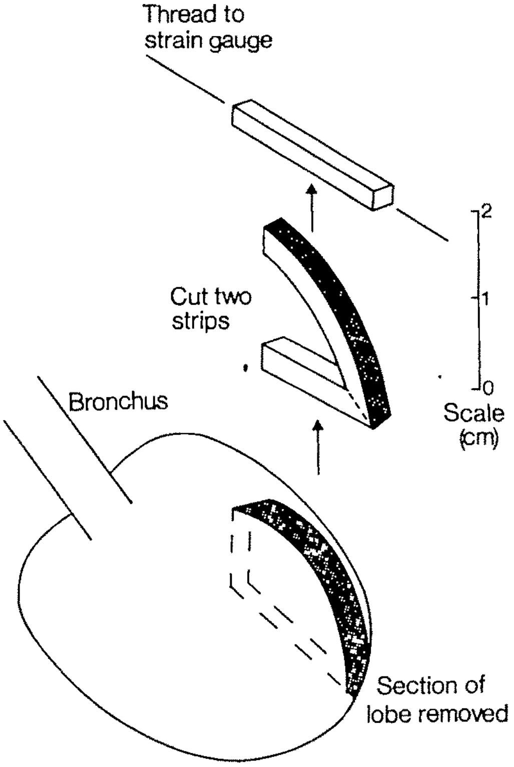


**Figure 1** Preparation of isolated strips from cat lung.

### *Preparation of antigenic extract from* Ascaris lumbricoides

*Ascaris lumbricoides* were removed from pigs; 200 g of the fresh worms were finely homogenized with 20 mM sodium phosphate buffer pH 7.4 at 4°C and made up to 500 ml. An equal volume of 0.85 M phenol solution was added, the mixture stirred for 1 h, centrifuged at 12,000 *g* for 10 min, and the supernatant dialysed for 48 h against running water and then for a further 12 h against distilled water containing 5 mM phosphate buffer at pH 7.4. The dialysed solution was then freeze-dried and yielded approximately 2 g of solid residue. This was stored under $N_2$ at −20°C.

### *Sensitization of cats to* Ascaris lumbricoides

Cats were injected subcutaneously with 5 mg of Ascaris extract in an equal volume of Freund's complete adjuvant every third day for 3 weeks. The cats were either manually restrained or lightly anaesthetized with halothane for this procedure. The animals were killed and the isolated lung strips and trachea prepared as above.

### *Calculations*

Mean concentration-response curves for the relaxing drugs were obtained by determining the concentration of drug which elicited a given percentage of the maximal response to (–)-isoprenaline. These concentration values for individual tissues were averaged and the standard error of the mean determined (Chahl & O'Donnell, 1967). The $EC_{50}$ value for a relaxing drug was calculated as the mean concentration giving 50% of its maximal response. Figures 2, 3 and 5 show examples of a typical experiment, a similar result being obtained in at least 4 other cats. $pA_2$ values were obtained by the method of Arunlakshana & Schild (1959) where at least 3 concentrations of antagonists were used for 20 min exposures in not less than 3 preparations. Statistical evaluation was performed using Student's $t$ test, $P < 0.05$ was regarded as significant.

### *Drugs*

The following drugs were used: (–)-isoprenaline hydrochloride (Sigma), (–)-adrenaline hydrochloride (Sigma), (±)-noradrenaline bitartrate (Sigma), histamine acid phosphate (Koch-Light), acetylcholine chloride (Roche), carbachol (Abbot Labs, injection), salbutamol sulphate (Allen & Hanbury's), terbutaline sulphate (Astra), compound 48/80 (Sigma), prostaglandins $E_1$ and $F_{2\alpha}$ (Upjohn), atropine sulphate (DHA), mepyramine maleate (May & Baker), methysergide (Sandoz), propranolol hydrochloride (ICI), practolol (ICI), phentolamine (Ciba), aspirin, indomethacin (Merck, Sharp & Dohm), flufenamate (Parke-Davis), bradykinin (Sigma), cocaine hydrochloride, normetanephrine hydrochloride (Calbiochem), Freund's complete adjuvant (CSL) and aminophylline (DHA).

Concentrations are expressed as either the free acid or base unless otherwise stated.

## Results

### *Isolated lung strip tone*

Isolated lung strips exhibited an intrinsic tone which was maintained for at least 6 hours. This tone could be relaxed by either aminophylline (10 µM to 10 mM) or catecholamines. Figure 2 shows examples of relaxation responses to (–)-isoprenaline. Relaxation was usually slow in onset, taking 5–10 min to reach completion. Following washout, the tissues recovered their initial tone within 30 minutes. Consistent responses could be elicited repeatedly at 30 min intervals.

Lung strips were contracted by histamine (0.1 µM to 0.1 mM) and prostaglandin $F_{2\alpha}$ (0.1 µM to 0.1 mM), examples of which are shown in Figure 2. Similar responses were elicited by acetylcholine (0.1 µM to 1.0 mM), compound 48/80 (0.01–1 mg/ml), potassium depolarizing solution and alternating current field stimulation (10 V/cm, 50 Hz for 0.8 seconds). Prostaglandin $E_1$ (0.1 µM to 10 µM) did not affect the tone of the lung strip (Figure 2).

The intrinsic tone of the lung strip depended on the initial tension applied to it (Figure 3). When the preparation was lightly stretched to give an initial tension of 1 g, (–)-isoprenaline (1 µM) elicited a small relaxation, typically of approx. 0.3 g in magnitude, but with greater stretch applied (–)-isoprenaline elicited a much larger relaxation. At tension of 5.0 g, the (–)-isoprenaline-induced relaxation was 2.3 grams. Since adequate responses to both contracting and relaxing durgs could be obtained with an initial tension of 2.0 g, this value was used for subsequent experiments.

The steady tone of lung strip was unaffected following exposure to atropine (1 µM), mepyramine (1 µM) or methysergide (10 µM) for 20 minutes. The tone of the preparation was not influenced by incubation for 1 h with aspirin (3 mM) or indomethacin (0.3 mM). However, sodium flufenamate from 50 µM to 0.5 mM produced a slow decrease in tone within 1 min of exposure. This relaxation was incomplete and in 4 preparations tested an average of 75% reduction in tone occurred after 30 min compared with the relaxation elicited by a maximal concentration of (–)-isoprenaline (1 µM).

### *Sensitivity of the isolated lung strip compared with the isolated trachea*

The pharmacology of the lung strip was different from that of the trachea. Histamine (0.1 µM to 0.1 mM) and prostaglandin $F_{2\alpha}$ (0.1 µM to 0.1 mM) strongly contracted the lung strip but the trachea was insensitive at these concentration ranges (Figure 2). Pretreatment with mepyramine (10 µM) completely prevented these histamine responses. Similarly, contractions induced with histamine (0.1 mM) were abolished by the subsequent addition of 10 µM mepyramine. Whereas prostaglandin $E_1$ (0.1 µM to 10 µM) had no effect on the intrinsic tone of the lung strip it relaxed the carbachol-induced tone of trachea. Bradykinin had no effect on either the lung strip or the trachea at concentrations up to 10 µg/ml.

(–)-Isoprenaline and (–)-adrenaline were much more potent in relaxing the lung strip than the trachea. The differences in the relaxant potencies of catecholamines on lung strip and trachea are shown in the concentration-response curves (Figure 4). (–)-Adrenaline was

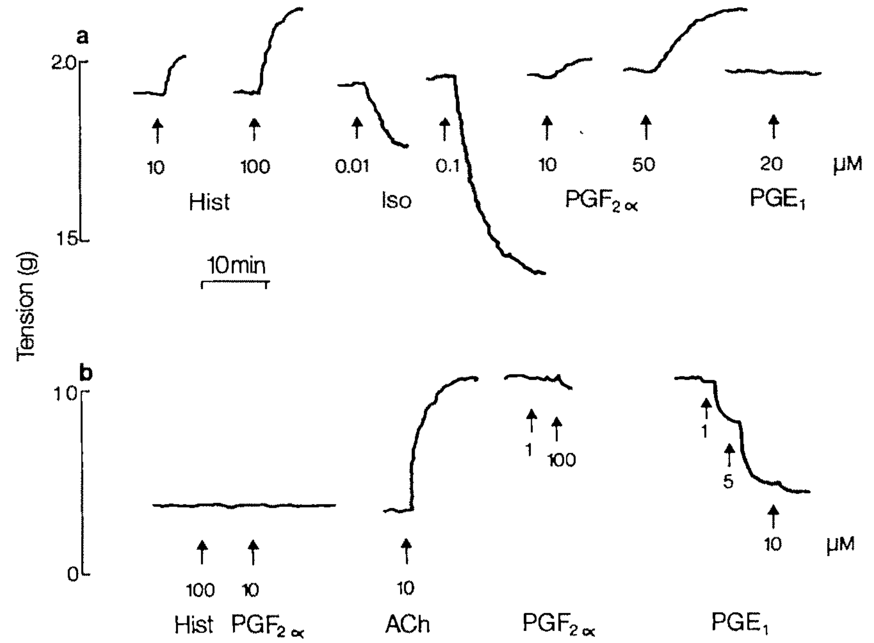


**Figure 2** Isometric tension responses of (a) isolated lung strip and (b) trachea of the cat; (a) lung strips: consecutive responses of one strip to histamine (Hist), (–)-isoprenaline (Iso), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin $E_1$ ($PGE_1$). An initial tension of 1.9 g was applied to the tissue by gently stretching. After each drug treatment the tissue was washed frequently and allowed to return to baseline tension before the next treatment. (b) Trachea: no response to histamine and prostaglandin $F_{2\alpha}$. When partially contracted by acetylcholine (ACh 10 μM) prostaglandin $F_{2\alpha}$ is still without effect, but prostaglndin $E_1$ causes relaxation. Concentrations μM.

72 times more potent than (±)-noradrenaline in lung strip whereas in trachea they were equipotent. The similar potencies of (–)-adrenaline and (±)-noradrenaline could not be explained by a dominant $Uptake_1$ mechanism occurring in the lung strip since cocaine (10 μM) did not significantly affect the $pD_2$ of (±)-noradrenaline (4 strips from 2 cats). Nor could an enhanced $Uptake_2$ mechanism in the trachea explain the similar potencies of (–)-adrenaline and (±)-noradrenaline since normetanephrine (10 μM) did not significantly shift the $pD_2$ values of (–)-adrenaline in

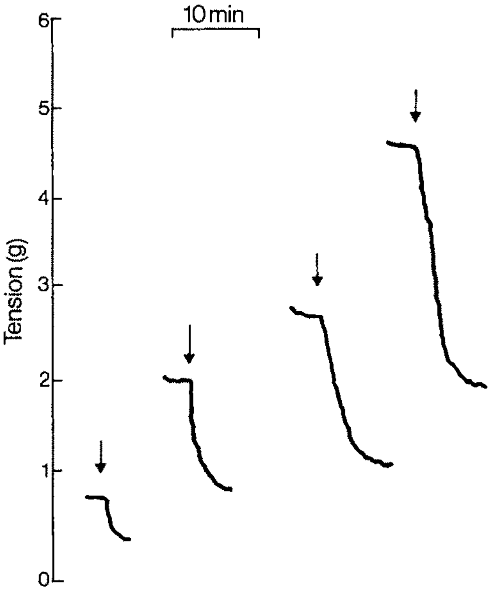


**Figure 3** The relationship between the initial tension applied to the isolated lung strip of the cat and the relaxation response to (–)-isoprenaline 1 μM (at arrow). A typical experiment in which a relaxation to (–)-isoprenaline was elicited and after washout the tissue was allowed to return to baseline then stretched to a greater resting tension. When this had stabilized the tissue was again challenged. The approximate initial tensions shown are 1,2,3 and 5 grams. Duplicate responses were elicited at the beginning and end of the experiment to check reproducibility.

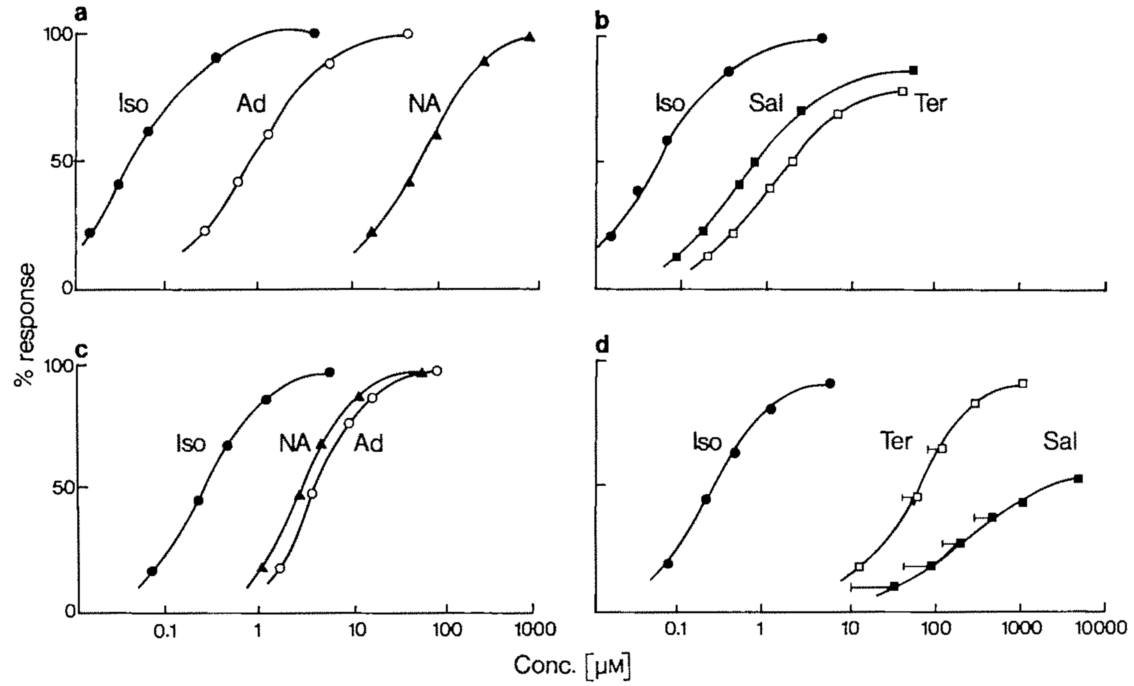


**Figure 4** Mean concentration-response curves of the isolated lung strip (a & b) and the trachea (c & d) of the cat to the catecholamines (–)-isoprenaline (Iso), (–)-adrenaline (Ad) and (±)-noradrenaline (NA) and the $\beta_2$-adrenoceptor selective agonists salbutamol (Sal) and terbutaline (Ter). Relaxation responses are plotted as percent of relaxation to a maximal concentration of (–)-isoprenaline on the intrinsic tone of the lung strip, and of the carbachol-induced tone in the trachea using carbachol (0.4 μM). Each point is the mean of at least 5 different tissues and the standard error bars are only shown when they exceed the dimensions of the symbols used.

trachea (4 tissues from 2 cats). $pD_2$ values, relative potencies and intrinsic activities of the catecholamines, terbutaline and salbutamol are shown in Table 1.

The different potencies of (–)-adrenaline and (±)-noradrenaline in lung strip, compared with trachea, suggested a difference in the type of β-adrenoceptor present and support for this was obtained by the use of the $\beta_2$-adrenoceptor selective agonists, salbutamol and terbutaline and the β-adrenoceptor antagonists, propranolol and practolol. For a 50% response the dose of salbutamol was about 10 times greater than that of isoprenaline when the lung strip was used, and

**Table 1** Table showing potency, $pD_2$, and intrinsic activity (α) of (–)-isoprenaline, (–)-adrenaline, (±)-noradrenaline, terbutaline and salbutamol on isolated lung strip and trachea of the cat

| | *Lung strip* | | | *Trachea* | | |
|---|---|---|---|---|---|---|
| | *Potency** | *$pD_2$†* | *α‡* | *Potency* | *$pD_2$* | *α* |
| (–)-Isoprenaline | 1000 | 7.34±0.02 (16) | 1 | 1000 | 6.74±0.25 (26) | 1 |
| (–)-Adrenaline | 52.2 | 6.07±0.03 (5) | 1 | 43.5 | 5.46±0.02 (13) | 1 |
| (±)-Noradrenaline | 0.75 | 4.22±0.04 (5) | 1 | 72.5 | 5.53±0.03 (16) | 1 |
| Terbutaline | 49.7 | 6.05±0.08 (10) | 0.77±0.02 | 4.5 | 4.59±0.15 (12) | 1 |
| Salbutamol | 93.4 | 6.34±0.06 (7) | 0.83±0.02 | 0.9 | 4.11±0.23 (9) | 0.62±0.05 |

*Potencies are the ratio of $pD_2$ values compared with that for (–)-isoprenaline which has been arbitrarily assigned a value of 1000. † The $pD_2$ value is defined as the negative log $EC_{50}$. Mean±s.e. mean. Number of tissues shown in brackets. ‡ Intrinsic activity for an agonist (Van Rossum, 1963) is expressed as the maximum response relative to that for (–)-isoprenaline. Mean ±s.e. mean.

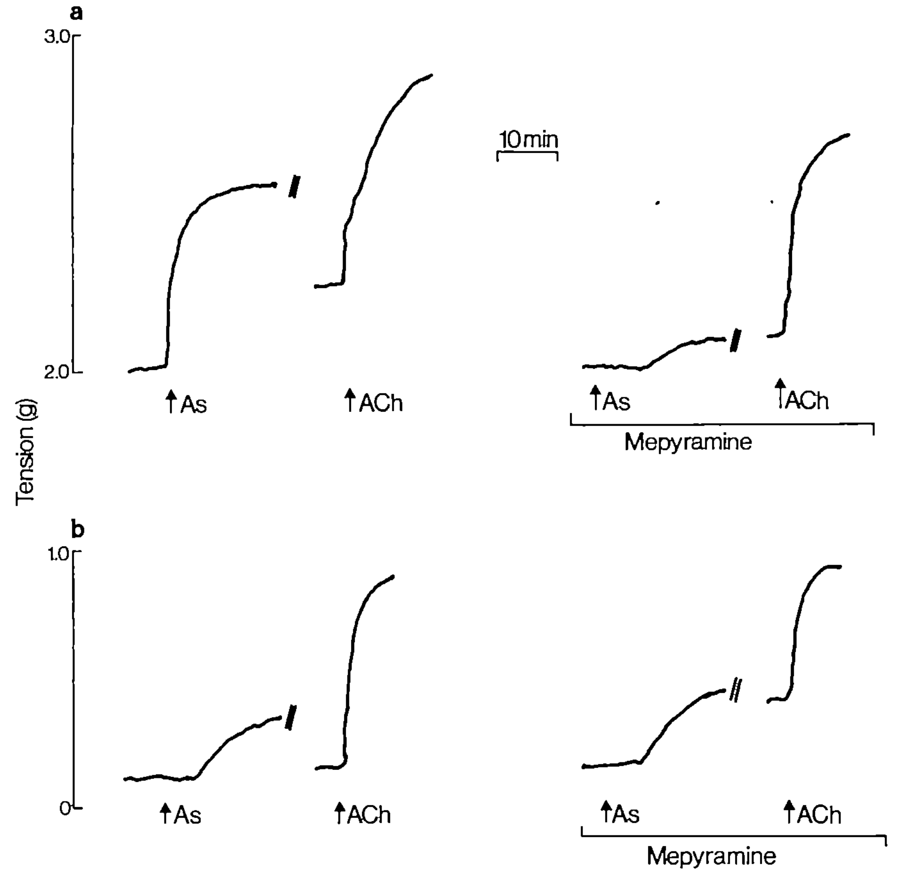


**Figure 5** Contractions elicted by *Ascaris* extract (1 mg/ml) of (a) the isolated lung strip and (b) trachea from a cat sensitized with *Ascaris* antigen (As). The upper and lower figures on the left show control responses to *Ascaris* extract. The lung strip contracts immediately to *Ascaris* extract but there is a delay of 5 min before the tracheal response is seen. On the right another pair of tissues is shown which were pre-treated for 5 min with mepyramine (100 µM). The immediate response of the lung strip is abolished and a smaller delayed contraction is seen but the tracheal response is maintained. In each case the magnitude of the contractile responses is compared with the tension developed to a maximal concentration of acetylcholine (ACh 100 µM) injected 10 min after washout of the *Ascaris* extract.

about 430 times greater for the trachea (Figure 4). Propranolol showed about equal potency in inhibiting (–)-isoprenaline responses of both the lung strip and trachea ($pA_2$ values of 8.0 and 8.1 respectively), whereas practolol was much less effective in inhibiting lung strip than trachea ($pA_2$ of 5.4 and 6.4, respectively).

The presence of α-adrenoceptors in the lung strip could not be readily demonstrated with α-adrenoceptor blockers. In four preparations, relaxation caused by (±)-noradrenaline (0.1 mM) was unchanged by the presence of phentolamine (30 µM). However, after β-adrenoceptor blockade using 20 min exposures of propranolol (10 µM) the response to (±)-noradrenaline (0.1 mM) was changed to a contraction averaging about 40% of the maximal response to acetylcholine (1 mM) (14 preparations from 5 cats). By contrast, tracheal preparations from the same cats gave contractions of only 10 to 15% of the acetylcholine maximum, and only 8 of the 14 tissues responded. Excitatory α-adrenoceptor participation in the trachea therefore seemed to be of minor significance, a finding which is in agreement with Fleisch, Maling & Brodie (1970).

### *Anaphylaxis of isolated lung strip and trachea*

*Ascaris* extract (1 mg/ml) elicited strong contractions (Schultz-Dale) in both lung strips and trachea from actively sensitized cats (Figure 5), but tissues from unsensitized animals were unresponsive. The response of the lung strip occurred immediately following challenge, but there was a consistent 2–8 min delay before the trachea contracted. This delay period was observed at all concentrations of *Ascaris* used (0.01–10 mg/ml).

The immediate phase of contraction of the lung strip could be abolished by exposure to mepyramine (1 to 100 μM) for 5 min prior to challenge (Figure 5). Response was then delayed for several minutes and resembled the tracheal contraction in this characteristic. Mepyramine (1 to 100 μM) added when anaphylactic concentrations had reached a plateau resulted in a 50–68% ($n=3$) reduction in tension in the lung strip in the ensuing 5 min, whereas the tracheal contractions were unchanged.

The Schultz–Dale contraction relaxed to baseline tension within about 20 min after washout of antigen. After contact with antigen, the tissue did not respond to compound 48/80 (50–100 μg/ml), but still responded normally to acetylcholine. Desensitization developed rapidly to the *Ascaris* extract, so that it was only possible to challenge each tissue once. Considerable variation in the magnitude of the anaphylactic responses to the *Ascaris* extract was found among the actively sensitized cats. Table 2 shows concentration-response data from three cats where a marked difference in the sensitivities of the tissues is seen to *Ascaris* extract in concentrations from 0.01–10 mg/ml.

## Discussion

We have described a new preparation whereby the direct effect of drugs on the small airways of the lungs can be examined *in vitro*. It seems probable that the contractile responses of the lung strip were due principally to activation of the airways smooth muscle. The lung strip chiefly comprises the spongy respiratory tissue of the lung lobe where the smooth muscle is present in alveolar ducts, respiratory bronchioles, bronchioles and blood vessels. Low power microscopic examination showed that the bronchioles had diameters of approx. 0.1 mm. From the sparsity of these bronchioles in the parenchyma, usually less than 2 per mm$^2$ cross section, it would appear that the smooth muscle of the alveolar ducts and respiratory bronchioles may make the major contribution to the contractile responses of the lung strip, transmitting tension throughout the strip via connective tissue linkages. A component of tone from vascular smooth muscle could not be demonstrated because firstly, (±)-noradrenaline caused relaxation which would not have been expected had the drug been acting principally on pulmonary vascular smooth muscle (Houghton & Phillips, 1975), and secondly, blockade of α-adrenoceptors with phentolamine did not enhance this (±)-noradrenaline-induced relaxation. After β-adrenoceptor blockade, (±)-noradrenaline in high concentration caused contraction of lung strips from which the existence of α-adrenoceptors may be inferred, but the extent to which they are located on bronchial or vascular smooth muscle is unknown.

The isolated lung strip preparation responded to a variety of stimuli. The responses were reproducible over several hours at 37°C and were slow to reach completion, resembling those of the isolated tracheal chain preparation (Castillo & De Beer, 1947). The presence of intrinsic tone was demonstrated by the ability of drugs such as the catecholamines and flufenamate to cause relaxation of strips. The fenamates in concentrations from 1–20 μg/ml have been shown to antagonize the actions of spasmogens such as slow reacting substance of anphylaxis (SRS-A) and prostaglandin $F_{2\alpha}$ on human bronchi (Collier & Sweatman, 1968), concentrations which are 10 times less than that required to relax cat lung strip. Indomethacin did not relax the intrinsic tone of cat

**Table 2** Comparison of anaphylactic contractions elicited by *Ascaris* extract on the isolated lung strip and trachea of three actively sensitized cats

| | *Cat 1* | | *Cat 2* | | *Cat 3* | |
|---|---|---|---|---|---|---|
| Ascaris *extract (mg/ml)* | *Lung strip* | *Trachea* | *Lung strip* | *Trachea* | *Lung strip* | *Trachea* |
| 0.01 | 43* | 63 | 0 | 0 | 10 | 2 |
| 0.1 | 50 | 57 | 14 | 2 | 15 | 6 |
| 1.0 | 68 | 61 | 42 | 17 | 14 | 7 |
| 10.0 | 106 | 62 | 74 | 31 | 17 | 11 |

*Response expressed as the percentage of the peak isometric tension elicited by a maximal concentration of ACh (1 mM).

lung strip although at concentrations of the order of 1 μM, it relaxes guinea-pig isolated trachea (Farmer, Farrar & Wilson, 1974). This may reflect a different profile of mediators responsible for the intrinsic tone of lung strip. The lack of effect of atropine, mepyramine and methysergide indicated that acetylcholine, histamine or 5-hydroxytryptamine were not involved in the maintenance of resting tone in the cat lung strip.

Histamine did not affect cat trachea, whereas contractions were elicited in lung strips. These histamine responses were abolished by mepyramine, indicating that the receptors were of the $H_1$ type (Black, Duncan, Durant, Gannelin & Parson, 1972). These results with lung strip provide *in vitro* evidence for an action of histamine on peripheral airways consistent with the observations of Colebatch (1970), who demonstrated a reduced lung compliance to histamine in anaesthetized cats. We have also observed differential sensitivity of the cat airways to the prostaglandins, prostaglandin $F_{2\alpha}$ contracting lung strips but not trachea, and prostaglandin $E_1$ relaxing trachea but not lung strip. It will be of interest to see whether this differential sensitivity of the central and peripheral airways to prostaglandins is seen using *in vivo* techniques which measure airways resistance and dynamic compliance (Widdicombe, 1963).

(–)-Isoprenaline, (–)-adrenaline and (±)-noradrenaline relaxed both trachea and lung strip preparations. The order of potency of the catecholamines on lung strip was found to be (–)-isoprenaline > (–)-adrenaline > (±)-noradrenaline, and this is the same order of potency as found in the guinea-pig isolated trachea, a $\beta_2$-adrenoceptor preparation (Farmer & Coleman, 1970; O'Donnell, 1972; O'Donnell & Wanstall, 1974). However, on the cat isolated trachea the order of potency was found to be (–)-isoprenaline > (±)-noradrenaline $\geqslant$ (–)-adrenaline. It was reported by Lands, Arnold, McAuliff, Luduena & Brown (1967) and Furchgott (1967) that (±)-noradrenaline is equipotent or more potent than adrenaline in eliciting a $\beta_1$-adrenoceptor response, so that the order of potencies of the catecholamines on the cat trachea is consistent with a $\beta_1$-adrenoceptor preparation. In support of this, the selective $\beta_2$-adrenoceptor agonists terbutaline and salbutamol were found to be, respectively 7 and 43 times more potent in relaxing the lung strip than the trachea. With propranolol, the non-selective $\beta$-adrenoceptor antagonist, identical $pA_2$ values were obtained against isoprenaline on both the lung strip and trachea, whereas practolol, the selective $\beta_1$-adrenoceptor antagonist (Dunlop & Shanks, 1968) was 10 times more active on trachea than the lung strip. These results support the conclusion that the adrenoceptors in the lung strip are predominantly the $\beta_2$ type and those of cat trachea $\beta_1$.

The ability to actively sensitize cats, in this instance to *Ascaris* extract, has not to our knowledge been reported elsewhere. Previous workers using antigens such as horse serum were unable to obtain active sensitization (Akcasu, 1962). We have consistently found both lung and trachea contract following antigen challenge *in vitro* with the *Ascaris* extract. The early part of the response of the lung strip was abolished with mepyramine, indicating that this phase was due to the release of histamine. The late response of the trachea and the mepyramine-treated lung strip demonstrated the release of a non-histamine component which is tentatively attributed to slow reacting substance of anaphylaxis (SRS-A), since prostaglandins $E_1$ and $F_{2\alpha}$ and bradykinin did not provoke contraction of the trachea. This differential sensitivity of the isolated lung and trachea of the cat may be useful for following the temporal release of mediators during anaphylaxis.

The isolated lung strip preparation has several advantages over conventional *in vitro* methods. It uses that part of the tracheobronchial tree which contributes most to the changes in compliance observed using *in vivo* techniques (Konzett & Rossler, 1940; Widdicombe, 1963). Furthermore, the preparation exhibits an active tone against which bronchodilator drugs may be tested, whereas the cat isolated trachea does not. The size of the organ permits a dozen or more tissues to be obtained from a set of lungs, thereby providing an adequate number of control and test strips. Insofar as this preparation has been tested to date, its usefulness as a model for investigation of pharmacological control of small airway function is indicated.

We wish to thank Professor H.J.H. Colebatch, of the Department of Medicine, University of New South Wales, for suggesting to us the use of the lung strip preparation, and Dr J. Pike of the Upjohn Company, for a gift of the prostaglandins. This work was supported by grants from the Asthma Foundation of Western Australia and the University Research Grants Committee of the University of Western Australia.

## References

AKCASU, A. (1959). The physiologic and pharmacologic characteristics of the tracheal muscle. *Archs int. Pharmacodyn. Ther.*, **122**, 201–207.

ARUNLAKSHANA, D. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac., Chemother.*, **14**, 48–58.

BLACK, J.W., DUNCAN, W.A.M., DURANT, C.J., GANNELIN, C.R. & PARSONS, E.M. (1972). Definition and antagonism of histamine $H_2$ receptors. *Nature, New Biol.*, **236**, 383–390.

CASTILLO, J.C. & DE BEER, E.J. (1947). The tracheal chain. *J. Pharmac. exp. Ther.*, **90**, 104–109.

CHAHL, L.A. & O'DONNELL, S.R. (1967). The interaction of cocaine and propranolol with catecholamines on guinea-pig trachea. *Eur. J. Pharmac.*, **2**, 77–82.

COLEBATCH, H.J.H. (1970). *Airway Dynamics: Physiology and Pharmacology*. ed. Bouhuys, A. pp. 169–189. London: C.C. Thomas.

COLLIER, H.O.J. & SWEATMAN, W.J.F. (1968). Antagonism by fenamates of prostaglandin $F_{2\alpha}$ and of slow reacting substance on human bronchial muscle. *Nature, London,* **219**, 864–865.

DUNLOP, D. & SHANKS, R.G. (1968). Selective blockade of adrenoreceptive beta receptors in the heart. *Br. J. Pharmac.*, **32**, 201–218.

DRAZEN, J.M. & AUSTEN, K.F. (1974). Effects of intravenous administration of slow reacting substance of anaphylaxis, histamine, bradykinin and prostaglandin $F_{2\alpha}$ on pulmonary mechanics in the guinea pig. *J. clin. Invest.*, **53**, 1679–1685.

FARMER, J.B. & COLEMAN, R.A. (1970). A new preparation of the isolated intact trachea of the guinea pig. *J. Pharm. Pharmac.*, **22**, 46–50.

FARMER, J.B., FARRAR, D.G. & WILSON, J. (1974). Antagonism of tone and prostaglandin mediated responses in a tracheal preparation by indomethacin and SC-19220. *Br. J. Pharmac.*, **52**, 559–565.

FLEISCH, J.H., MALING, H.M. & BRODIE, B.B. (1970). Evidence for the existence of alpha-adrenergic receptors in the mammalian trachea. *Am. J. Physiol*, **218**, 596–599.

FURCHGOTT, R.F. (1967). The pharmacological differentiation of adrenergic receptors. *Ann. N.Y. Acad. Sci.*, **139**, 553–570.

HOUGHTON, J. & PHILLIPS, E.M. (1973). The pharmacology of human isolated pulmonary vascular tissue. *Br. J. Pharmac.*, **47**, 676–677.

KONZETT, H. & ROSSLER, R. (1940). Versuchanordung zu Untersuchungen an der Bronchial-muskulatur. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **195**, 71–74.

LANDS, A.M., ARNOLD, A., McAULIFF, J.P., UDUENA, F.P. & BROWN, T.C. (1967). Differentiation of receptor systems activated by sympathomimetic amines. *Nature, Lond.*, **214**, 597–598.

O'DONNELL, S.R. (1972). An examination of some β-adrenoreceptor stimulants for selectivity using the isolated trachea and atria of the guinea pig. *Eur. J. Pharmac.*, **19**, 371–379.

O'DONNELL, S.R. & WANSTALL, J.C. (1974). Potency and selectivity *in vitro* of compounds related to isoprenaline and orciprenaline on β-adrenoreceptors in the guinea pig. *Eur. J. Pharmac.*, **52**, 407–417.

SPARROW, M.P. & MITCHELL, H.W. (1975). Pharmacological characterization of the isolated cat lung strip—an *in vitro* preparation of fine airways. *Proc. Sixth Int. Congr. Pharmac.*, Helsinki. Abstract 378.

VAN ROSSUM, J.M. (1963). Cumulative dose—response curves. *Arch. int. Pharmacodyn.*, **143**, 299–329.

WIDDICOMBE, J.G. (1963). Regulation of tracheobronchial smooth muscle. *Physiol. Rev.*, **43**, 1–37.

*(Received December 8, 1975.*
*Revised March 30, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 81–88

# THE EFFECT OF HIGH RATES OF VASOPRESSIN ADMINISTRATION ON RENAL POTASSIUM AND SODIUM EXCRETION DURING POTASSIUM LOADING IN THE SHEEP

A.M. BEAL
Department of Physiology, University of Queensland, St. Lucia, Queensland,
Australia and the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

1 The influence of potassium loading on the renal excretion of sodium, potassium and solute during high rate vasopressin administration has been investigated in sheep.

2 Adrenalectomized sheep were infused with 0.43 M KCl at 2 ml/min for 2–2.5 hours. Coincident with the rise in plasma potassium concentration, the urinary excretion of sodium, potassium, solute and water was increased as was the reabsorption of solute-free water. The rates of urinary excretion of sodium and potassium, osmolal clearance ($C_{Osm}$) and solute-free water reabsorption ($T^c_{H_2O}$) for the first 50 min of potassium infusion were each found to be linearly related to the plasma potassium concentration.

3 After 50 min an infusion of vasopressin at 1 or 4 mu/min was superimposed on the potassium infusion for a period of 30 minutes. The administration of vasopressin was consistently associated with further augmentation of potassium excretion and clearance, of osmolal clearance and of solute-free water reabsorption to values above those anticipated from the pre-vasopressin regression lines for these parameters. Urinary sodium showed a coincident depression in the rate of excretion and clearance during the same period.

4 Thirty to fifty minutes after the cessation of vasopressin infusion the potassium and sodium excretions had returned to values which approximated the pre-vasopressin relations between plasma potassium and the urinary excretions of these ions.

5 Both rates of vasopressin infusion were equally effective in increasing the potassium clearance. Any differences in clearance between the two rates of vasopressin administration were not statistically significant.

6 The large increments in potassium excretion (averaging > 40%) were interpreted as indicating that, when vasopressin is present at high concentrations, the distal tubule is one site of action of the hormone in the nephron of sheep.

## Introduction

It has been known for some time that, in addition to altering water reabsorption by the kidney, vasopressin can influence the rate of renal excretion of the monovalent ions, sodium, potassium and chloride. However, the degree to which vasopressin alters the renal excretion of these electrolytes varies considerably with the most consistent effects occurring during water diuresis. In the water-loaded dog vasopressin increases the rate of renal sodium excretion but frequently does not increase potassium excretion (Shannon, 1942; Anslow & Wesson, 1955; Ali, 1958; Brooks & Pickford, 1958; Chan & Sawyer, 1961; Perlmutt, 1961). A similar response to vasopressin has been observed in the rat (Sawyer, 1952; Chan, 1965) although in one report (Heller & Stephenson, 1950) increased excretion of potassium was associated with reduced sodium excretion. In contrast vasopressin administration to sheep undergoing water diuresis usually causes increased potassium excretion with the effects on sodium excretion being the more variable (Kinne, Macfarlane & Budtz-Olsen, 1961; Cross, Thornton & Twedell, 1963; Kuhn & Peeters, 1965). The renal electrolyte response has proved to be even less reliable when vasopressin was given to animals in anti-diuresis. Non-hydrated dogs have frequently shown no response at all whereas sheep in the same state have responded to vasopressin administration with increased excretion of sodium, potassium and chloride (Kinne *et al.*, 1961; Kuhn & Peeters, 1965).

Clearly vasopressin can increase the renal excretion of electrolytes but which ion or ions will be increased cannot be reliably predicted. This variability of response suggests that vasopressin does not specifically control the renal excretion of any of these ions and that the effect is dependent on some factor or factors associated with the relative availability of the ions at the time of observation. In a recent study Scott & Morton (1976) found that high level vasopressin administration to sodium-loaded sheep invariably increased renal sodium excretion with little effect on potassium excretion. The object of the experiments described in this paper was to determine whether a potassium-loaded sheep would respond to vasopressin by increasing the renal excretion of potassium rather than that of sodium.

## Methods

### *Experimental procedures*

The experiments were performed on 4 Merino ewes weighing 30–35 kg and 4 Clun Forest ewes weighing 37–57 kg. All animals were bilaterally adrenalectomized in 2 separate operations and after the second operation the sheep were allowed a minimum recovery period of 4 weeks before being used for experiment. The adrenalectomized sheep were maintained with intramuscular injections of 1.5–2.0 mg deoxycorticosterone acetate (DOCA) and 8–15 mg cortisone given once daily at 17 h 00 min. The sheep were allowed 1000 g chaff daily with continual access to water and were provided with a salt lick composed of sodium bicarbonate, chloride and phosphate. At 17 h 00 min on the day before the experiment any uneaten food was removed but access to salt and water was maintained until the experiment started.

Before each experiment a catheter (Casper pattern: Rusch) was inserted into the urinary bladder. This procedure was assisted by the application of a topical anaesthetic solution ('Xylocaine' spray; Astra Chemicals Ltd., Watford) to the vulva and urethral orifice and by lubricating the tip of the catheter with sterile anaesthetic jelly ('Xylocaine' Gel; Astra Chemicals Ltd., Watford). Both jugular veins were cannulated under local anaesthesia (lignocaine 3% with adrenaline 1:50,000; Willows Francis) using the technique of Seldinger (1953). The sheep were then restrained on leather stretchers in a normal upright position with their feet just off the floor. All solutions were given by intravenous infusion at 2 ml/min into one jugular cannula with blood samples being collected from the other cannula into tubes or syringes heparinized with one drop of heparin (5000 iu/ml). Urinary collections were made according to standard clearance techniques; clearance periods were of 10 min duration with blood samples being taken mid-period. After collection of a sample of urine and plasma (control period) the animals were primed with 20 ml of a solution containing 5 g inulin and 6 g sodium para-amino hippurate (PAH)/100 ml given via the infusion cannula. The animals were now allowed a 2 h stabilization period during which a solution containing 1.5 g inulin and 0.6 g PAH/100 ml of 0.125 M NaCl was infused. At the end of this infusion three clearance collections were made. Thereafter a solution containing 1.5 g inulin and 0.6 g PAH/100 ml of 0.43 M KCl was infused for the remaining 2–2.5 h of experiment. After 50 min of KCl infusion, vasopressin (Pitressin; Parke Davis or arginine vasopressin; Sigma) was infused for 30 min at one of the following rates: (1) All four Merino sheep were given an infusion of 4 mu/min Pitressin (pressor units) after a loading injection of 10 mu. (2) Two of the Clun Forest sheep were infused with arginine vasopressin at 4 mu/min (pressor units) without any loading injection. (3) All four Clun Forest sheep were infused with arginine vasopressin at 1 mu/min without any loading injection.

### *Analytical procedures*

The following analyses were performed on the samples of urine and plasma:

(1) Inulin was estimated in urine and plasma by either the method of Davidson & Sackner (1963) or the method of Heyrovsky (1956) adapted to the Technicon autoanalyzer by Dawborn (1965).

(2) PAH was estimated in urine and plasma by either the method of Smith, Finkelstein, Aliminosa, Crawford & Graber (1945) or the method of Bratton & Marshall (1939) as adapted for the Technicon autoanalyzer by Harvey & Brothers (1962).

(3) Osmolality was calculated from the freezing-point depression as measured with either an Advanced osmometer (Model 31 LAS) or a Fiske osmometer (Model G 62).

(4) Sodium and potassium were estimated in diluted urine and plasma with an E.E.L. flame photometer (Model A) using mixed standards in the appropriate concentration ranges for the samples.

### *Mathematical and statistical procedures*

The values for the renal plasma clearances of inulin, PAH and electrolytes were calculated as follows:

$$\text{Plasma clearance} = \frac{\text{urinary excretion rate}}{\text{plasma concentration}}$$

Solute-free water reabsorption = osmolal clearance − urine flow rate.

As Beal & Harrison (1975) have observed the concentration of solutes in the urine collected from a bladder catheter does not alter as soon as the urine flow alters because there is a finite volume or 'dead-space' between the kidney and the exterior in the

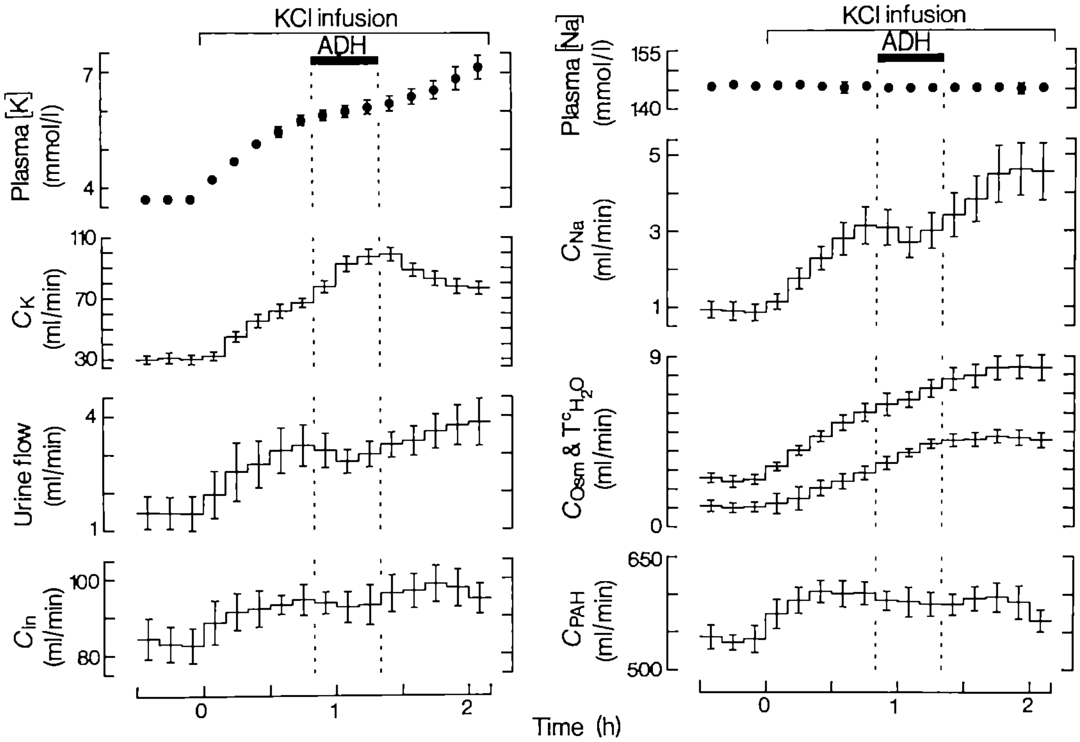


Figure 1 The concentrations of potassium and sodium in plasma, urine flow, solute-free water reabsorption ($T^c_{H_2O}$) and the clearance of potassium ($C_K$), sodium ($C_{Na}$), solute ($C_{Osm}$), inulin ($C_{In}$) and para-amino hippurate (PAH) ($C_{PAH}$) before, during and after vasopressin infusion (1 mu or 4 mu/min) into adrenalectomized sheep being potassium loaded at 860 µmol/minute. The solid bars indicate the period of vasopressin administration. Mean results are given; vertical lines show s.e. mean.

urinary tract and catheter. This 'dead-space' volume tends to cause errors in the measurement of rates of urinary excretion of solutes particularly when the urine collection times are short and the flow rate is changing rapidly. As the excretion rate of inulin is affected in a similar manner to the other urinary solutes, the excretion rates of potassium and sodium, the osmolal clearance and the solute-free water reabsorption were expressed as a ratio of the inulin clearance to prevent 'dead-space' errors from affecting the interpretation of these results. These parameters are presented in this form in Figures 2 and 3.

The slopes of linear regressions were compared by *t* test of regression coefficients. Analysis of covariance was applied to the potassium data for the period during which vasopressin was infused at 1 mu or 4 mu/minute. The covariates used in the analysis were the potassium clearances for the 2 periods immediately preceding vasopressin administration.

## Results

As the infusion of vasopressin at 1 mu/min and 4 mu/min produced similar changes in renal function, all experiments have been summarized as a single treatment. During the infusion of potassium chloride the plasma potassium concentration was elevated and the concentration of sodium either remained unchanged or fell slightly. The concentrations of sodium and potassium in the urine rose as did the urine flow, resulting in increased excretions and clearances of sodium and potassium. These changes were associated with augmented osmolal clearance and solute-free water reabsorption. Coincident with the increased electrolyte excretion the inulin and PAH clearances also rose with the maximum rates of increase in these clearances occurring during the first 30 min of potassium infusion (Figure 1). Since the increases in potassium and sodium excretion rates,

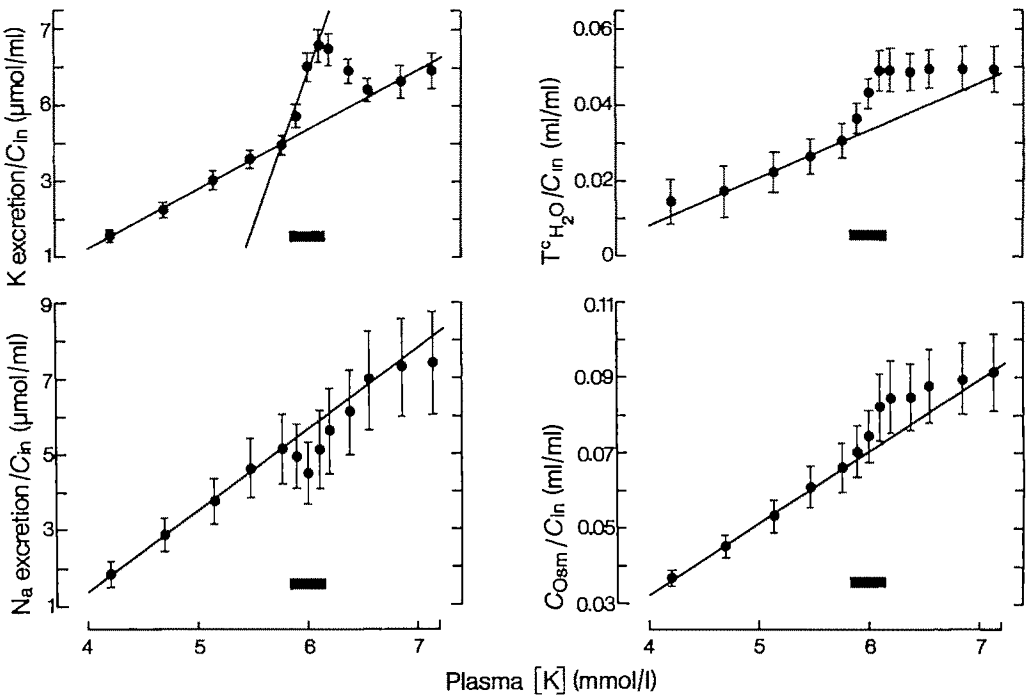


**Figure 2** Regressions of urinary potassium excretion, urinary sodium excretion, solute-free water reabsorption ($T^c{}_{H_2O}$) and osmolal clearance ($C_{Osm}$) on the plasma potassium concentration before, during and after vasopressin infusion (1 mu or 4 mu/min) into adrenalectomized sheep being potassium-loaded at 860 μmol/minute. Each of the urinary parameters has been expressed as a ratio of the inulin clearance ($C_{in}$). The solid bars indicate the period of vasopressin administration. The calculated regression lines for the mean data for the 50 min (5 clearance periods) before vasopressin infusion are shown and are extended to higher plasma potassium concentrations as lines of prediction for the period during and after vasopressin infusion. Mean results are given; vertical lines show s.e. mean.

osmolal clearance and solute-free water reabsorption were obviously correlated with the progressive increases in plasma potassium concentration these parameters were plotted against the plasma potassium concentration and found to be linearly related to the plasma potassium (Figure 2).

The administration of vasopressin caused the urine flow to fall slightly in some experiments whereas in the remainder, flow continued to rise at a slower rate. The clearances of inulin and PAH also fell slightly in some experiments, usually, though not always, in the same experiments in which urine flow fell. After the vasopressin infusion had stopped the urine flow and the clearances of inulin and PAH resumed increasing and only started to fall at high plasma potassium concentrations.

During the infusion of vasopressin the urinary concentration and rate of excretion of potassium were further augmented and the rate of rise in plasma potassium concentration slowed, resulting in an increase in the slope for the regression of urinary potassium excretion on plasma potassium in all experiments. The regression lines for urinary potassium excretion on plasma potassium concentration for the periods before and during vasopressin infusion were compared in individual experiments by *t* test of regression coefficients and in all experiments the increase in slope during vasopressin administration was statistically significant (*P* ranged from 0.05 to 0.001). On cessation of vasopressin administration the urinary potassium concentration and excretion rate fell and the rate of rise in plasma potassium concentration increased again so that after 30–50 min the relation between potassium excretion and plasma concentration approximated that occurring before vasopressin infusion (Figures 2 and 3).

In contrast to potassium the concentration of sodium in the urine fell during vasopressin administra-

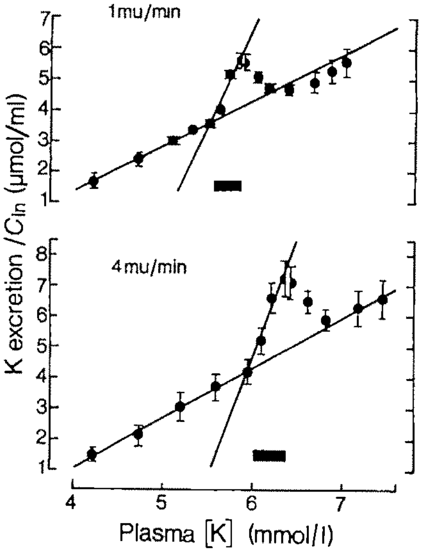


**Figure 3** Regression of urinary potassium excretion on plasma potassium concentration before, during and after the infusion of vasopressin at 1 mu/min (4 sheep) and at 4 mu/min (6 sheep) into adrenalectomized sheep being potassium loaded at 860 µmol/minute. The data for potassium excretion are expressed as a ratio of the inulin clearance ($C_{In}$). The solid bars indicate the period of vasopressin administration. The calculated regression lines for the mean data for the 50 min before vasopressin infusion and for the 30 min during vasopressin infusion are shown. Mean results are given; vertical lines show s.e. mean.

tion in 8 of the experiments and the excretion of sodium decreased in all experiments to values below those expected from the pre-vasopressin regression of sodium excretion on plasma potassium concentration. The extent of this fall in urinary sodium excretion was variable and on a molar basis, the decline in sodium excretion was never greater and was frequently less than the increment in potassium excretion for the same clearance period. After the vasopressin infusion had ended the rate of sodium excretion rose to such an extent that the pre-vasopressin relation between urinary sodium excretion and plasma potassium concentration appeared to be re-established (Figure 2).

Superficially the increase in urinary potassium excretion in the group receiving 1 mu/min vasopressin appeared to be smaller than that for the sheep receiving 4 mu/min (Figure 3). However, when the ratios of potassium clearance/inulin clearance for the 3 clearance periods of vasopressin infusion were compared by analysis of covariance, no significant differences were found between the 2 rates of vasopressin infusion ($F_{2:6}=4.32$, 0.31, 0.02 respectively).

## Discussion

As Thorn (1968) observed, one of the difficulties in the interpretation of the effect of neurohypophysial hormones on renal electrolyte excretion has been that the status of water and salt metabolism of the experimental animals has not been well defined or controlled. Although the overall water and mineral balances of the sheep in the present investigation were not uniform, the animals were all in positive potassium balance as a result of the potassium infusion. In the intact animal hyperkalaemia has been shown to increase aldosterone release from the adrenal gland (Funder, Blair-West, Coghlan, Denton, Scoggins & Wright, 1969). By performing the present experiments on adrenalectomized sheep the possible intervention of endogenous adrenal steroids released as a result of the potassium infusion was prevented. As judged from salivary sodium/potassium ratios the effect of the DOCA maintenance injection reaches maximum and returns to sodium-replete ratios within 6–8 h of injection (Beal, Budtz-Olsen, Clark, Cross & French, 1975) so that during these experiments which started 18–20 h after the maintenance injection the concentrations of circulating steroids from exogenous sources would be expected to be low and falling relatively slowly.

The increase in plasma potassium concentration during the infusion of potassium chloride into the sheep was always associated with increased urinary excretion of both sodium and potassium. The rate of excretion of each ion was found to be linearly related to the plasma potassium concentration provided that renal function is not altered by other factors. Micropuncture studies in rat and dog have shown that during potassium loading, the reabsorption of sodium and water in the proximal tubule is depressed thus providing an explanation for the natriuretic diuresis observed in hyperkalaemia (Malnic, Klose & Giebisch, 1966; Watson, 1966; Brandis, Keyes & Windhager, 1970). Observations in potassium-infused sheep although less direct, suggest that the same explanation is tenable for the increase in urinary sodium excretion in this species also (Beal, Budtz-Olsen, Clark, Cross & French, 1973; Beal & Harrison, 1975).

The infusion of vasopressin resulted in a significant increase in the excretion of potassium and a coincident decrease in the excretion of sodium. The site of action of vasopressin on water permeability and electrolyte transport appears to be restricted to the nephron segments distal to the loop of Henle. In dogs and rats vasopressin does not alter the water permeability of the proximal convoluted tubule (Clapp, Watson & Berliner, 1963; Gertz, Kennedy & Ullrich, 1964; Ullrich, Rumrich & Fuchs, 1964) or the loop of Henle (Wirz, 1956; Gottschalk, 1961; Morgan & Berliner, 1968). Likewise Davis, Knox & Berliner (1967) found no evidence of sodium reabsorption in the proximal tubule being altered by vasopressin although some earlier reports suggest that vasopressin might stimulate sodium reabsorption in this segment (Clapp *et al.*, 1963; Gottschalk, cited by Gertz *et al.*, 1964). In the rat various lines of evidence indicate that the distal convoluted tubule is one site of action of vasopressin on water reabsorption (Wirz, 1956; Gottschalk & Mylle, 1959; Darmady, Durant, Matthews & Stranack, 1960) but in other species such as dog, monkey and *Meriones* the distal tubule does not show the same responsiveness to the hormone (Clapp & Robinson, 1966; Bennett, Brenner & Berliner, 1968; Rouffignac, Lechene, Guinnebault & Morel, 1969). The collecting duct is therefore the only unequivocal site of action of vasopressin on water permeability in the mammalian kidney. Although the infusion of a hyperosmotic solution into sheep would be expected to raise plasma vasopressin concentrations, maximum antidiuretic activity had not been achieved in most experiments as is evidenced by the increment in solute-free water reabsorption resulting from the administration of vasopressin being larger than the corresponding increase in osmolal clearance. However, much of the increment in solute-free water reabsorption clearly results from the addition of solute to the tubular fluid. When vasopressin was infused renal potassium excretion was significantly augmented. Under some conditions particularly potassium loading, secretion of potassium occurs in the collecting duct although in the rat, it adds no more than a small percentage of the total potassium excretion (Hierholzer, 1961; Malnic *et al.*, 1966; Diezi, Michoud, Aceves & Giebisch, 1973). In the present investigation the size of the increment in potassium excretion which averaged over 40% in excess of the rate predicted from the pre-vasopressin regression line (Figure 2) indicates that unless the collecting duct of the sheep has a much greater capacity to secrete potassium than that of the rat, the main site for this effect must be the distal tubule as no other segment of the nephron has the secretory capability consistent with such an increase in potassium excretion.

The fall in renal sodium excretion during vasopressin infusion may be explained by one of a number of mechanisms. The inulin clearance fell slightly in some experiments during vasopressin infusion. However, it is doubtful if this represents a real fall in glomerular filtration rate (GFR) since in most cases the urine flow fell also and the urinary inulin concentration rose after a short delay which suggested that this fall in inulin clearance was a 'dead-space' effect resulting from a fall in urine flow during short urine collection periods. Expression of the sodium excretion as a ratio of the inulin clearance should correct for this artifact. In other experiments the inulin clearance was not depressed indicating that the fall in sodium excretion rate was not dependent on a fall in GFR. Since the fall in urinary sodium excretion was not due to a fall in GFR the lowering of sodium excretion during vasopressin infusion must be the result of increased reabsorption of sodium in the nephron. If vasopressin acts directly on the sodium pump of the cortical collecting duct as some evidence suggests (Morgan & Berliner, 1968; Helman, Grantham & Burg, 1971; Frindt & Burg, 1972) the fall in sodium excretion may be due to increased active reabsorption in this segment. However, if Ullrich, Baldamus, Uhlich & Rumruch (1969) are correct in their conclusion that vasopressin increases the sodium permeability of the collecting duct rather than affecting active transport, the reduction in sodium excretion may result from the influx of potassium into the tubule reducing the potential difference across the tubule thus increasing the ease with which sodium could be reabsorbed from this segment. These arguments may extend to the distal tubule of the sheep as well but as yet no direct measurements of the effect of vasopressin on water permeability or sodium transport in the nephron have been reported for this species. The situation is obviously confused by the continuing increase in plasma potassium concentration causing ever increasing amounts of sodium to be rejected in the proximal segment of the nephron.

Following the cessation of vasopressin infusion there was an obvious return of the relationship between plasma potassium concentration and the excretions of sodium and potassium to the condition which existed before vasopressin administration although the continuous fall in steroid levels throughout the experiment may have led to a slight change in this relationship (Figure 2). At still higher plasma potassium levels both potassium and sodium excretion began to fall as the haemodynamic state of the kidney was affected by the high plasma potassium concentration.

The effects on urinary potassium excretion of Pitressin and arginine-vasopressin were of similar magnitude which agrees with the observation of Macfarlane, Kinne, Walmsley, Siebert & Peter (1967) that arginine-vasopressin, lysine-vasopressin and Pitressin were of equal effect in the adult sheep. Although the two rates of infusion of vasopressin used in these experiments were clearly pharmacological the similarity of the response to these dose rates suggests that they were both supramaximal and that administration of vasopressin at more physiological rates would also augment urinary potassium excretion during potassium loading. The results obtained in this work support the thesis that relative availability of sodium and potassium is one important determinant of the pattern of change in renal excretion of these ions during vasopressin administration.

I wish to thank Mrs M. Ford and Mr P. Burrow for their technical assistance with these experiments.

## References

ALI, M.M. (1958). A comparison of some activities of arginine-vasopressin and lysine-vasopressin on kidney functions in conscious dogs. *Brit. J. Pharmac. Chemother.*, **13**, 131–137.

ANSLOW, W.P. & WESSON, L.G. (1955). Some effects of pressor-antidiuretic and oxytocic fractions of posterior pituitary extract on sodium, chloride, potassium and ammonium excretion in the dog. *Am. J. Physiol.*, **182**, 561–566.

BEAL, A.M. & HARRISON, F.A. (1975). Renal function in sheep during infusion of alkali metal ions into the renal artery. *J. Physiol., Lond.*, **245**, 137–162.

BEAL, A.M., BUDTZ-OLSEN, O.E., CLARK, R.C., CROSS, R.B. & FRENCH, T.J. (1973). Renal and salivary responses to infusion of potassium chloride, bicarbonate and phosphate in Merino sheep. *Q. Jl exp. Physiol.*, **58**, 251–265.

BEAL, A.M., BUDTZ-OLSEN, O.E., CLARK, R.C., CROSS, R.B. & FRENCH, T.J. (1975). Changes in renal haemodynamics and electrolyte excretion during acute hyperkalaemia in conscious adrenalectomized sheep. *Q. Jl exp. Physiol.*, **60**, 207–221.

BENNETT, C.M., BRENNER, B.M. & BERLINER, R.W. (1968). Micropuncture study of nephron function in the Rhesus monkey. *J. clin. Invest.*, **47**, 203–216.

BRANDIS, M., KEYES, J. & WINDHAGER, E.E. (1970). Potassium-induced inhibition of proximal tubular fluid reabsorption. *Physiologist, Wash.*, **13**, 154.

BRATTON, A.C. & MARSHALL, E.K. (1939). A new coupling component for sulphanilamide determination. *J. biol. Chem.*, **128**, 537–550.

BROOKS, F.P. & PICKFORD, M. (1958). The effect of posterior pituitary hormones on the excretion of electrolytes in dogs. *J. Physiol., Lond.*, **142**, 468–493.

CHAN, W.Y. (1965). Effects of neurohypophysial hormones and their deamino analogues on renal excretion of sodium, potassium and water in rats. *Endocrinology*, **77**, 1097–1104.

CHAN, W.Y. & SAWYER, H. (1961). Saluretic actions of neurohypophysial peptides in conscious dogs. *Am. J. Physiol.*, **201**, 799–803.

CLAPP, J.R. & ROBINSON, R.R. (1966). Osmolality of distal tubular fluid in the dog. *J. clin. Invest.*, **45**, 1847–1853.

CLAPP, J.R., WATSON, J.F. & BERLINER, R.W. (1963). Osmolality, bicarbonate concentration and water reabsorption in proximal tubule of the dog nephron. *Am. J. Physiol.*, **205**, 273–280.

CROSS, R.B., THORNTON, W.M. & TWEDELL, E.D. (1963). The effect of vasopressin on water and electrolyte excretion by the sheep. *Aust. J. exp. Biol. med. Sci.*, **41**, 629–636.

DARMADY, E.M., DURRANT, J., MATTHEWS, E.R. & STRANACK, F. (1960). Location of $^{131}$I Pitressin in the kidney by autoradiography. *Clin. Sci.*, **19**, 229–241.

DAVIDSON, W.D. & SACKNER, M.A. (1963). Simplification of the anthrone method for determination of inulin in clearance studies. *J. lab. clin. Med.*, **62**, 351–356.

DAVIS, B.B., KNOX, F.G. & BERLINER, R.W. (1967). The effect of vasopressin on proximal tubule sodium reabsorption in the dog. *Am. J. Physiol.*, **212**, 1361–1364.

DAWBORN, J.K. (1965). Application of Heyrovsky's inulin method to automatic analysis. *Clin. Chim. Acta*, **12**, 63–66.

DIEZI, J., MICHOUD, P., ACEVES, J. & GIEBISCH, G. (1973). Micropuncture study of electrolyte transport across papillary collecting duct of the rat. *Am. J. Physiol.*, **224**, 623–634.

FRINDT, G. & BURG, M.B. (1972). Effect of vasopressin on sodium transport in renal cortical collecting tubules. *Kidney Int.*, **1**, 224–231.

FUNDER, J.W., BLAIR-WEST, J.R., COGHLAN, J.P., DENTON, D.A., SCOGGINS, B.A. & WRIGHT, R.D. (1969). Effect of plasma [K+] on the secretion of aldosterone. *Endocrinology*, **85**, 381–384.

GERTZ, K.H., KENNEDY, G.C. & ULLRICH, K.J. (1964). Mikropunktionuntersuchungen über die Flüssigkeitsrückresorption aus den einzelnen Tabulusabschnitten bei Wasserdiurese (diabetes insipidus). *Pflügers Arch. ges. Physiol.*, **278**, 513–519.

GOTTSCHALK, C.W. (1961). Micropuncture studies of tubular function in the mammalian kidney. *Physiologist, Wash.*, **4**, 35–55.

GOTTSCHALK, C.W. & MYLLE, M. (1959). Micropuncture study of the mammalian urinary concentrating mechanism: evidence for the countercurrent hypothesis. *Am. J. Physiol.*, **196**, 927–936.

HARVEY, R.D. & BROTHERS, A.J. (1962). Renal extraction of paraamino-hippurate and creatinine measured by continuous *in vivo* sampling of arterial and renal vein blood. *Ann. N.Y. Acad. Sci.*, **102**, 46–54.

HELLER, H. & STEPHENSON, R.P. (1950). Effect of posterior pituitary extract and its fractions on renal electrolyte excretion. *Nature, Lond.*, **165**, 189.

HELMAN, S.I., GRANTHAM, J.J. & BURG, M.B. (1971). Effect of vasopressin on electrical resistance of renal cortical collecting tubules. *Am. J. Physiol.*, **220**, 1825–1832.

HEYROVSKY, A. (1956). A new method for the determination of inulin in plasma and urine. *Clin. Chim. Acta*, **1**, 470–474.

HIERHOLZER, K. (1961). Secretion of potassium and acidification in collecting ducts of mammalian kidney. *Am. J. Physiol.*, **201**, 318–324.

KINNE, R., MACFARLANE, W.V. & BUDTZ-OLSEN, O.E. (1961). Hormones and electrolyte excretion in the sheep. *Nature, Lond.*, **192**, 1084–1085.

KUHN, E. & PEETERS, G. (1965). Influence de l'arginine vasopressin sur l'excrétion d'électrolytes chez le mouton. *Archs int. Pharmacodyn. Thér.*, **155**, 455–458.

MACFARLANE, W.V., KINNE, R., WALMSLEY, C.M., SIEBERT, B.D. & PETER, D. (1967). Vasopressin and the increase in water and electrolyte excretion by sheep, cattle and camels. *Nature, Lond.*, **214**, 979–981.

MALNIC, G., KLOSE, R.M. & GIEBISCH, G. (1966). Micropuncture study of distal tubular potassium and sodium transport in the rat kidney. *Am. J. Physiol.*, **211**, 529–547.

MORGAN, T. & BERLINER, R.W. (1968). Permeability of the loop of Henle, vasa recta and collecting duct to water, urea and sodium. *Am. J. Physiol.*, **215**, 108–115.

PERLMUTT, J.H. (1961). Renal activity of vasopressin in anesthetized dogs. *Am. J. Physiol.* **200**, 400–404.

ROUFFIGNAC, C. DE, LECHENE, C., GUINNEBAULT, M. & MOREL, F. (1969). Etude par microponction de l'élaboration de l'urine. III Chez le mérion non diurétique et en diurèse par le mannitol. *Nephron*, **6**, 643–666.

SAWYER, W.H. (1952). Posterior pituitary extracts and excretion of electrolytes by the rat. *Am. J. Physiol.*, **169**, 583–587.

SCOTT, D. & MORTON, J.J. (1976). Changes in urinary water and electrolyte excretion in sodium-loaded sheep in response to intravenous infusion of arginine vasopressin. *Q. Jl exp. Physiol.*, **61**, 57–70.

SELDINGER, S.I. (1953). Catheter replacement of the needle in percutaneous arteriography. *Acta Radiologica*, **39**, 368–376.

SHANNON, J.A. (1942). The control of renal excretion of water. *J. exp. Med.*, **76**, 387–399.

SMITH, H.W., FINKELSTEIN, N., ALIMINOSA, L., CRAWFORD, B. & GRABER, M. (1945). Renal clearances of substituted hippuric acid derivatives and other aromatics in man. *J. clin. Invest.*, **24**, 388–404.

THORN, N.A. (1968). The influence of the neurohypophysial hormones and similar polypeptides on the kidneys. In *Neurohypophysial Hormones and Similar Polypeptides, Handb. exp. Pharmac.* Vol. 23, ed. Berde, B. pp. 372–442. Berlin, Heidelberg & New York: Springer-Verlag.

ULLRICH, K.J., RUMRICH, G. & FUCHS, G. (1964). Wasserpermeabilität und transtubulärer Wasserfluss corticaler Nephronabschnitte bei verschiedener Diuresezuständen. *Pflügers Arch. ges. Physiol.*, **280**, 99–119.

ULLRICH, K.J., BALDAMUS, C.A., UHLICH, E. & RUMRICH, G. (1969). Influence of ionic calcium and antidiuretic hormone on transtubular sodium transport in the rat kidney. *Pflügers Arch.*, **310**, 369–376.

WATSON, J.F. (1966). Potassium reabsorption in the proximal tubule of the dog nephron. *J. clin. Invest.*, **45**, 1341–1348.

WIRZ, H. (1956). Der osmotische Druck in den corticalen Tubuli der Ratteniere. *Helv. Physiol. Pharmac. Acta*, **14**, 353–362.

*(Received January 28, 1976.*
*Revised March 24, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 89–100

# THE RELEASE OF A CORONARY VASODILATOR METABOLITE FROM THE GUINEA-PIG ISOLATED PERFUSED HEART STIMULATED BY CATECHOLAMINES, HISTAMINE AND ELECTRICAL PACING AND BY EXPOSURE TO ANOXIA

K.J. BROADLEY

Department of Applied Pharmacology, Welsh School of Pharmacy, University of Wales Institute of Science and Technology, King Edward VII Avenue, Cardiff CF1 3NU

1 A procedure involving two guinea-pig isolated hearts perfused in series is described for detecting in the recipient heart the release of a possible coronary vasodilator metabolite from the donor heart.

2 Adrenaline and isoprenaline stimulated the rate and force of contraction and produced a multiphasic coronary vascular response, the predominant phase of which was vasodilatation. When the $\beta$-adrenoceptors of the recipient heart were blocked, stimulation of the donor heart by the catecholamines was associated with a coronary vasodilatation of the recipient heart..

3 Histamine stimulated rate and force of contraction and was predominantly coronary vasodilator. After blockade of histamine $H_1$- and $H_2$-receptors in the recipient heart, coronary vasodilatation followed increases in activity of the donor heart in response to histamine.

4 These vasodilator responses of the recipient heart were attributed to the release from the donor heart of a vasodilator metabolite by the increased activity. This is the proposed mechanism for the predominant coronary vasodilator response to catecholamines and histamine.

5 Periods of electrically-paced tachycardia and anoxia of the donor heart also led to the release of vasodilator activity.

6 The possible identity of the metabolite is discussed.

## Introduction

The overall coronary vascular response to catecholamines is vasodilatation (Wégria, 1951; Charlier, 1961; Parratt, 1968; Dempsey & Cooper, 1972) which is comprised of several components arising from both direct and indirect effects. The direct effects on the coronary vasculature yield both vasoconstriction mediated via $\alpha$-adrenoceptors (Saito, 1959; Kaverina, 1965; Parratt, 1967; Broadley, 1970) and vasodilatation mediated via $\beta$-adrenoceptors differing from those of the myocardium (Adam, Boyles & Scholfield, 1970; Ross & Jorgensen, 1970; Bayer, Mentz & Förster, 1972) and therefore assigned to the $\beta_2$-type (Broadley, 1970; Parratt & Wadsworth, 1970; Mark, Abboud, Schmid, Heistad & Mayer, 1972; Ross, 1974). Others however disagree and claim that these resemble the $\beta_1$-adrenoceptors of the myocardium (Lucchesi & Hodgeman, 1971; Drew & Levy, 1972). The indirect effects result from the simultaneous positive inotropic and chronotropic responses, both of which restrict coronary flow by extravascular compression of the coronary vessels (Melville & Lu, 1950; Douglas, Armengol & Talesnik, 1960). The second indirect effect may account for the predominant vasodilator phase of the coronary vascular response. This component only occurs when there are significant changes in the rate and force of contraction and is reduced when these myocardial responses are antagonized (Hashimoto, Shigei, Imai, Saito, Yago, Uei & Clark, 1960; Siegal, Gilmore & Sarnoff, 1961). It is also prevented by metabolic inhibitors such as cyanide (Garcia-Ramos, Alanis & Luco, 1950) and fluoracetate (Saito, 1959) and is therefore attributed to the increased metabolic activity of the heart (Green, Wégria & Boyer, 1942; Melville & Lu, 1950; Douglas *et al.*, 1960; Hashimoto *et al.*, 1960; Hardin, Scott & Haddy, 1961; Siegal *et al.*, 1961; Berne, 1964). It has been suggested that a vasodilator metabolite is released by the myocardium during the increased activity (Berne, 1964; Haddy & Scott, 1968). Attempts to demonstrate such a metabolite in the venous outflow of hearts stimulated by adrenaline have been few, possibly because of interference by the simultaneous appearance of the adrenaline. Only the nucleoside, adenosine, has been

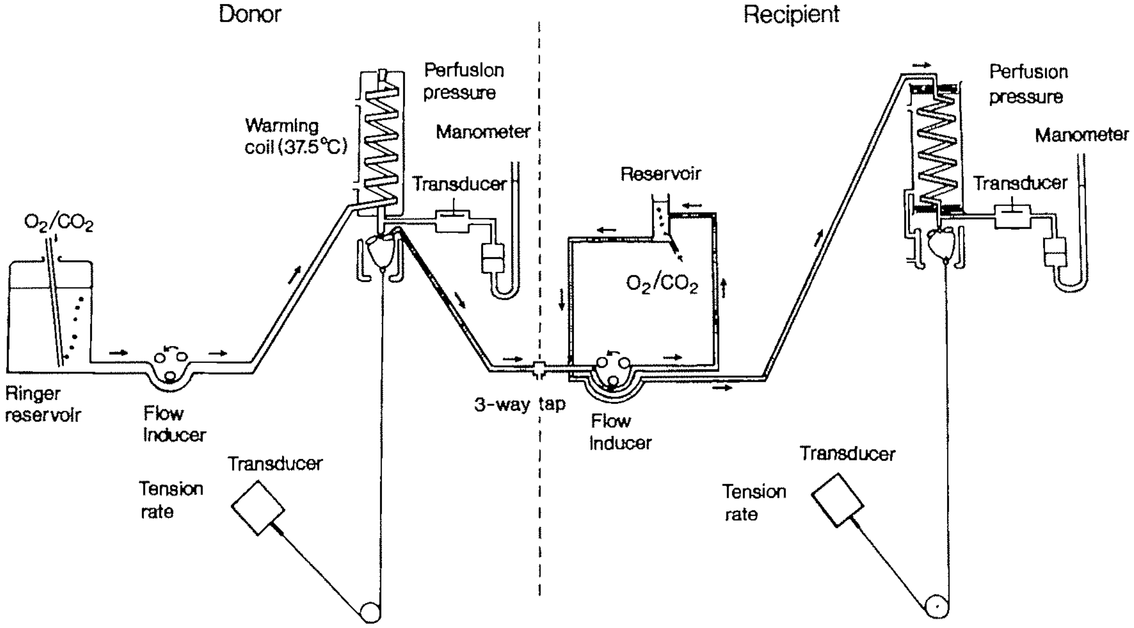


**Figure 1** Schematic diagram of the apparatus used to perfuse two guinea-pig isolated hearts in series, with the recipient heart served either with fresh Krebs-bicarbonate solution or the perfusate from the donor heart. Perfusion pressure and rate and force of contractions were recorded in each heart.

successfully detected (Katori & Berne, 1966). Most work to substantiate the claim of a putative mediator has in fact been based upon the modification of the vasodilatation by substances known to affect the proposed substance. In the case of adenosine this evidence tended to disprove its role since dipyridamole, an inhibitor of its uptake, failed to potentiate the vasodilatation to catecholamines (Parratt & Wadsworth, 1972).

The present study was undertaken to develop a technique whereby the presence of a vasodilator metabolite following cardiac stimulation by catecholamines could be demonstrated pharmacologically and that it indeed had coronary vasodilator activity. The principle of the procedure was to take the perfusate from an isolated perfused heart to supply a second heart which would thus respond to the presence of any released substance(s). The choice of an alternative drug with which to stimulate the heart was virtually limited to histamine by the availability of an effective antagonist of the myocardial and coronary vascular responses of the drug in the recipient heart.

The myocardial effects of histamine may be abolished by $H_2$-receptor antagonists such as burimamide (Black, Duncan, Durant, Ganellin & Parsons, 1972; McNeill & Verma, 1974; Levi, Capurro & Lee, 1975). These have also been shown to antagonize the coronary vasodilatation (Ercan, Bökesoy & Türker, 1974) which presumably arises indirectly from the increased myocardial activity (Broadley, 1975a). However, histamine also exerts direct vasodilator and constrictor effects upon the coronary vasculature and these are mediated via $H_1$-receptors (Broadley, 1975a). This may account for the demonstration by others of antagonism *of the overall* coronary vasodilatation by mepyramine (Levi & Kuye, 1974). Therefore both antagonists were required to prevent the effects of histamine in the recipient heart.

To avoid the problems of contamination of the perfusate by the agonist, increased activity of the donor heart was also induced by electrically-paced tachycardia. Finally, in view of the possibility that the increased myocardial activity produces a relative anoxia which may serve as an intermediate to the release of a metabolically-linked vasoactive metabolite (Berne, 1958), experiments were performed in which the donor heart was exposed to periods of anoxia to determine whether this too would release a vasodilator metabolite. A preliminary account of the technique used has been presented to the British Pharmacological Society (Broadley, 1975b).

## Methods

Guinea-pigs of either sex and weight range 275–675 g were killed by a blow on the head and the hearts and lungs together were rapidly excised and transferred to a beaker containing Krebs-bicarbonate solution. Donor hearts were set up initially. A small incision was made in the aorta which was tied onto a glass cannula for retrograde perfusion of the coronary vessels by a modified (Broadley, 1970) Langendorff (1895) method (Figure 1). The venae cavae and pulmonary artery and vein were tied off and only then were the lungs trimmed free. The pulmonary artery was cut which thus provided the only route of escape for the perfusate. A constant rate (5–6 ml/min) of perfusion was achieved by means of a Watson-Marlow flow inducer (MHRE/30/T, 0.5 mm tube bore). The Krebs-bicarbonate solution, having the following composition in g/l distilled water; NaCl 6.92, KCl 0.345, $CaCl_2 2H_2O$ 0.28, $NaHCO_3$ 2.1, $MgSO_4 7H_2O$ 0.29, glucose 2.0, $NaH_2PO_4 2H_2O$ 0.16, was gassed with 5% $CO_2$ in $O_2$ before passing through the flow inducer to the warming coil at the base of which was the cannulated aorta. Alterations in perfusion pressure (resting level between 25 and 50 mmHg; 1 mmHg $\approx$ 133 Pa) arising from changes in coronary vascular resistance were recorded on a Devices M19 polygraph by means of a pressure transducer (Bell & Howell, Type 4-327-L221) situated between the flow inducer and warming coil. Also located at this point was a Condon manometer.

Isometric contractions were recorded with a transducer (Ether, Type UF1, 114 g sensitivity range) attached via a pulley to a clip on the apex of the ventricles. The transducer was adjusted to apply a resting diastolic tension of 1–2 g on the heart. Rate of contraction was recorded by a ratemeter (Devices, Type 2751) triggered by the signal from the tension trace.

Drugs were injected into the perfusion solution through the connecting rubber tubing immediately prior to entry into the warming coil. The dose volume was between 0.025 and 0.2 ml and this produced a small injection artefact which was well separated from the drug response.

The recipient heart was then set up, intially perfused with fresh Krebs-bicarbonate solution taken from a reservoir via a 3-way tap. The perfusion apparatus was essentially the same, but certain additional features were required to satisfy the criteria necessitated by the very small quantities of possible metabolites likely to be released. All the perfusate had to be collected from the donor heart and taken immediately to the second heart, the perfusion rates of both hearts had to match exactly and the perfusate required regassing and rewarming. A reservoir was provided for this purpose and also to serve as a trap for any air arriving from the donor heart and a point where fresh Ringer solution and the drugs could be added. The volume of this reservoir and all the tubing had to be minimal to avoid dispersion and dilution of the small amounts of any metabolite. The perfusion pressure and tension and rate of contraction of the recipient heart were recorded similarly to the donor heart and both hearts were perfused and jacketed at 37°C.

After both hearts had stabilized, the cut pulmonary artery of the donor heart was cannulated with a length of polythene tubing and by turning the 3-way tap, the perfusate was taken to the recipient heart.

Electrically-induced tachycardia of donor hearts was produced by placing bipolar electrodes on the surface of the ventricles and stimulating with square-wave pulses (5 Hz, 10 V, 5 ms) delivered by an SRI stimulator (Type 6053) for periods of 2 minutes.

In the experiments where the donor heart was exposed to anoxia, the Krebs-bicarbonate solution was replaced by Locke solution having the following composition in g/l distilled water: NaCl 9.0, KCl 0.42, $CaCl_2$, 0.24, $NaHCO_3$ 0.5 and glucose 1.0. This was gassed with $O_2$ so that the omission of the gas would deprive the heart of only $O_2$ and not both $O_2$ and $CO_2$ which would occur had Krebs-bicarbonate solution been used.

Drugs used were: (–)-adrenaline acid tartrate (BDH Ltd.), burimamide (SK & F Labs Ltd.), histamine acid phosphate (Sigma), (–)-isoprenaline bitartrate dihydrate (Ward Blenkinsop Ltd.), mepyramine maleate (Anthisan, May & Baker Ltd.), phentolamine mesylate (CIBA), propranolol (ICI Ltd.). All solutions were freshly prepared in 0.9% w/v NaCl solution (saline) and amounts referred to in the text are expressed as the base.

## Results

With the two hearts satisfactorily connected in series and no loss of perfusate the effects of adrenaline were examined (Figure 2). Adrenaline (0.25 μg) added to the recipient heart reservoir increased the tension and rate of contraction and produced a characteristic coronary vascular response consisting of an initial rise in perfusion pressure, indicative of vasoconstriction, followed by a more prolonged vasodilatation. The same dose of adrenaline to the donor heart produced identical responses. This adrenaline was carried in the perfusate to the recipient heart where smaller responses were produced. These responses of the recipient heart to the adrenaline were sufficient to mask any effects of a metabolite arriving concurrently from the donor heart. The direct effects of adrenaline on the $\beta$-adrenoceptors of the recipient heart were therefore antagonized by the non-selective $\beta$-antagonist propranolol. This was used because it would block both the $\beta_1$-adrenoceptors responsible for the myocardial stimulation and the vascular $\beta_2$-adrenoceptors responsible for direct vasodilatation. Propranolol (15 μg) added to the recipient heart reservoir satisfactorily antagonized the positive

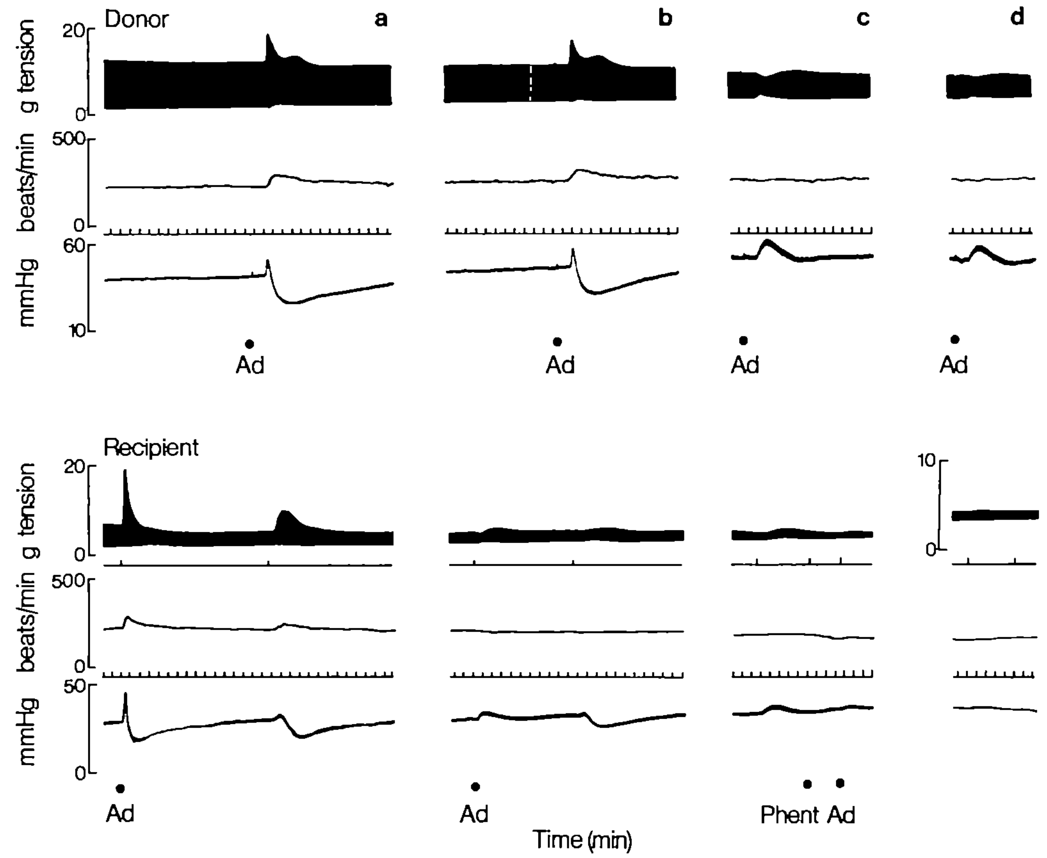


**Figure 2** Donor and recipient isolated perfused (5 ml/min) hearts from guinea-pigs (518 and 458 g respectively). The effects of adrenaline (Ad, 0.25 μg) on the contractile force (upper records), heart rate (middle records) and coronary perfusion pressure (lower records) of each heart are shown. Propranolol was added to the recipient heart reservoir (15 μg) between (a) and (b) and to the donor heart reservoir (10 μg) between (b) and (c). Phentolamine (Phent, 10 μg) was added to the recipient heart in (c).

inotropic and chronotropic responses to adrenaline together with the vasodilatation. Only a small vasoconstriction remained (Figure 2b). The repeated dose of adrenaline in the donor heart yielded unchanged responses, however after a short delay (1 min) the perfusate reaching the recipient heart produced a vasodilatation unaccompanied by rate or tension changes. The positive inotropic and chronotropic responses to adrenaline and the coronary vasodilatation in the donor heart were antagonized by introduction of propranolol (10 μg) to the perfusion solution; only a vasoconstriction remained (Figure 2c). The anticipated vasodilatation that had previously followed adrenaline administration to the donor heart was also abolished, although a small vasoconstriction occurred. Finally, the α-adrenoceptor antagonist, phentolamine (10 μg), was added to the reservoir and this antagonized the residual vasoconstriction to adrenaline, whether added to the donor or recipient heart (Figure 2c and d).

Since this vasoconstriction tended to oppose the vasodilator activity carried over from the donor heart, the experiments were repeated using isoprenaline which has negligible constrictor activity. Isoprenaline (10 ng) was added to the recipient and donor hearts to produce positive inotropic and chronotropic responses together with a predominant coronary vasodilatation (Figure 3a). The myocardial and coronary vascular β-adrenergic effects in the recipient heart were antagonized with propranolol (10 μg) and the responses to a repeated dose of isoprenaline in the donor heart were unchanged. On arrival at the recipient heart, where no direct β-adrenoceptor effects were possible, vasodilatation occurred (Figure 3b). This was reproducible with a second dose of isoprenaline. Antagonism of the tension and rate

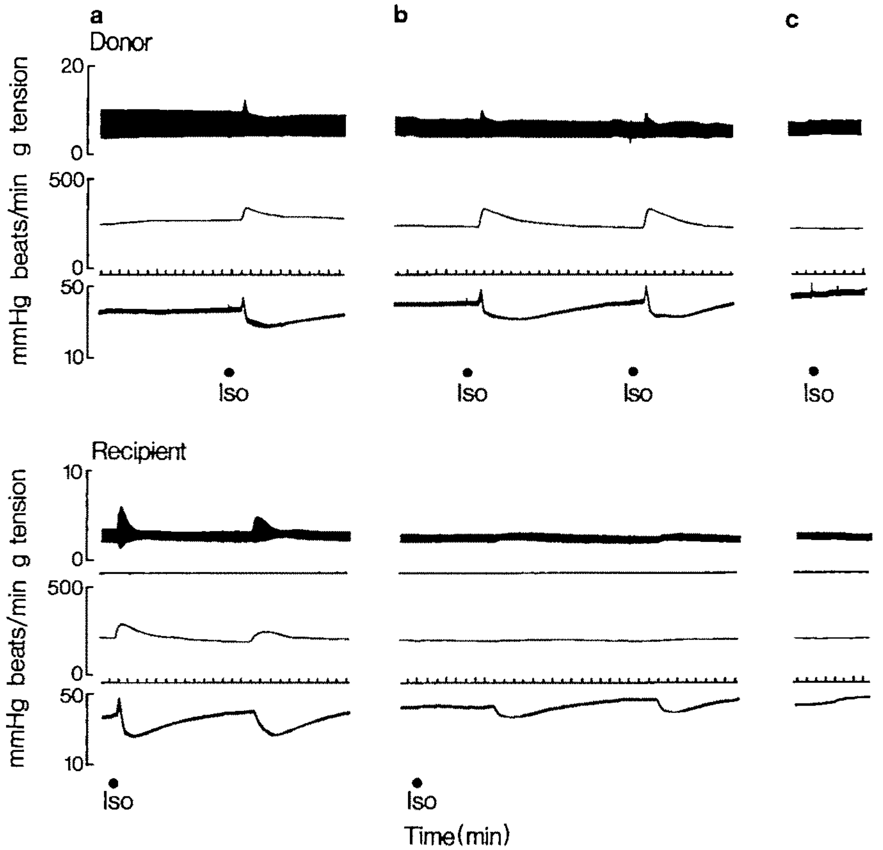


**Figure 3** Donor and recipient isolated perfused (5 ml/min) hearts from guinea-pigs (485 and 630 g respectively). The effects of isoprenaline (Iso, 10 ng) on the contractile force (upper records), heart rate (middle records) and coronary perfusion pressure (lower records) of each heart are shown. Propranolol was added to the recipient heart reservoir (10 µg) between (a) and (b) and to the donor heart reservoir between (b) and (c).

responses to isoprenaline in the donor heart by propranolol (10 µg) also abolished this vasodilatation in the recipient heart that had previously followed the isoprenaline response in the donor heart. Therefore both the catecholamines adrenaline and isoprenaline, in stimulating the donor heart, caused the appearance of vasodilator activity in the recipient heart where their direct effects were antagonized.

The study was next extended to determine whether cardiac stimulation by histamine would also lead to this vasodilator activity carried over to the second heart. Histamine (1 µg) was administered consecutively to the donor and recipient hearts (Figure 4a). Each heart exhibited positive inotropic and chronotropic responses and triphasic coronary vascular responses consisting of an initial fall in perfusion pressure followed by a rise in pressure and finally a more prolonged vasodilatation. The histamine added to the donor heart was carried over to the recipient heart which yielded smaller identical responses, and these, as with the catecholamines, masked any response to a possible metabolite. These effects of histamine on the second heart were antagonized by the combined addition to the reservoir of the $H_2$-receptor antagonist burimamide (2 mg) and the $H_1$-receptor antagonist mepyramine (5 µg). These antagonize the myocardial and vascular effects respectively and histamine repeated immediately produced no response. Because of the short-lived action of burimamide (Broadley, 1975a), this combined antagonist administration had to be repeated immediately before the subsequent histamine administration to the donor heart. This produced unchanged responses in the donor heart followed shortly by a vasodilatation in the recipient. Antagonism of the histamine responses in the donor

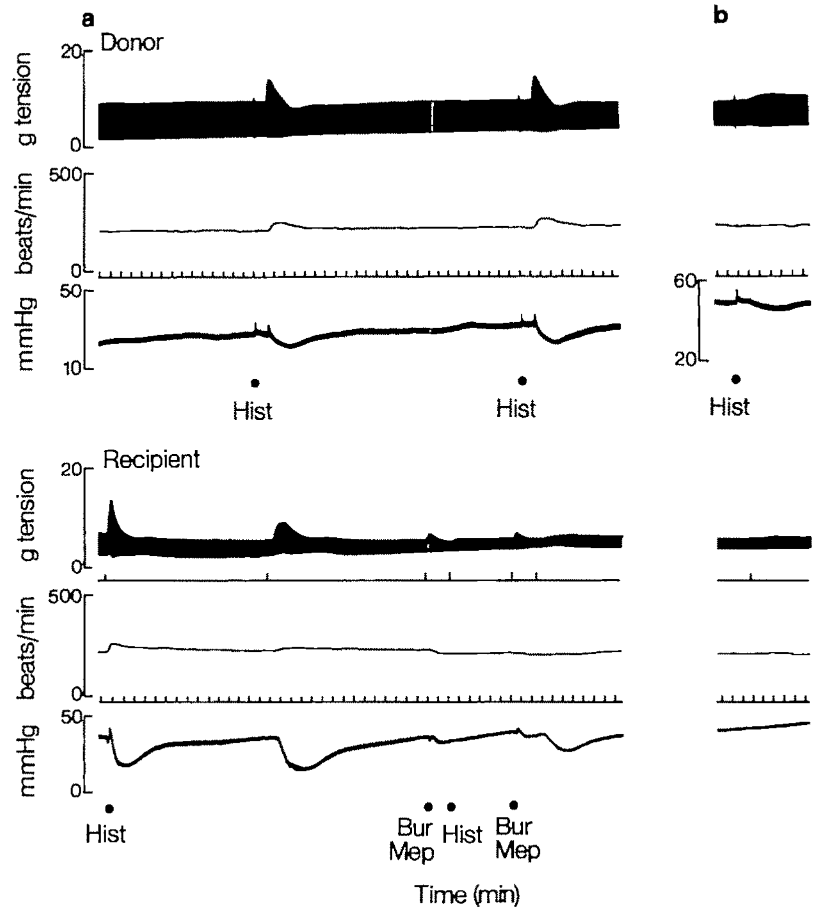


**Figure 4** Donor and recipient isolated perfused (6 ml/min) hearts from guinea-pigs (663 and 575 g respectively). The effects of histamine (Hist, 1 μg) on the contractile force (upper records), heart rate (middle records) and coronary perfusion pressure (lower records) of each heart are shown. Burimamide (Bur, 2 mg) and mepyramine (Mep, 5 μg) were added simultaneously to the recipient heart reservoir immediately before the following dose of histamine added to either the donor or recipient hearts in (a). The donor heart was perfused with burimamide (20 μg/ml) and mepyramine (1 μg/ml) during (b).

heart by perfusing burimamide (20 μg/ml) and mepyramine (1 μg/ml) also abolished this vasodilatation in the recipient heart (Figure 4b).

At this stage the burimamide in the perfusate to the donor heart was omitted and immediately the myocardial responses of both hearts to histamine started to return (Figure 5) and were completely restored within 16 minutes. By this time the vasodilatation had also returned although the continued presence of mepyramine prevented any direct vascular effects. Repeating the antagonism of myocardial effects in the recipient heart with burimamide (2 mg) again revealed the carriage over of vasodilator activity from the donor heart when stimulated by histamine (Figure 5). This demonstrated both the reproducibility and the persistence of the releasing capabilities of the donor heart over a prolonged period of time.

Tachycardia was induced into the donor hearts of another series of experiments. These hearts were paced with square wave pulses (10 V, 5 ms) at 5 Hz compared with their normal spontaneous rate of 210 beats/minute. This was the fastest possible rate that the hearts would follow. During the 2 min pacing, the force of contraction was diminished and there was a small vasoconstriction followed by a small vasodilatation (Figure 6a). Following this the recipient heart also produced a vasodilatation. However, this vasodilatation was small compared with those achieved with the catecholamines and histamine-induced tachycardia. Although several stimulation

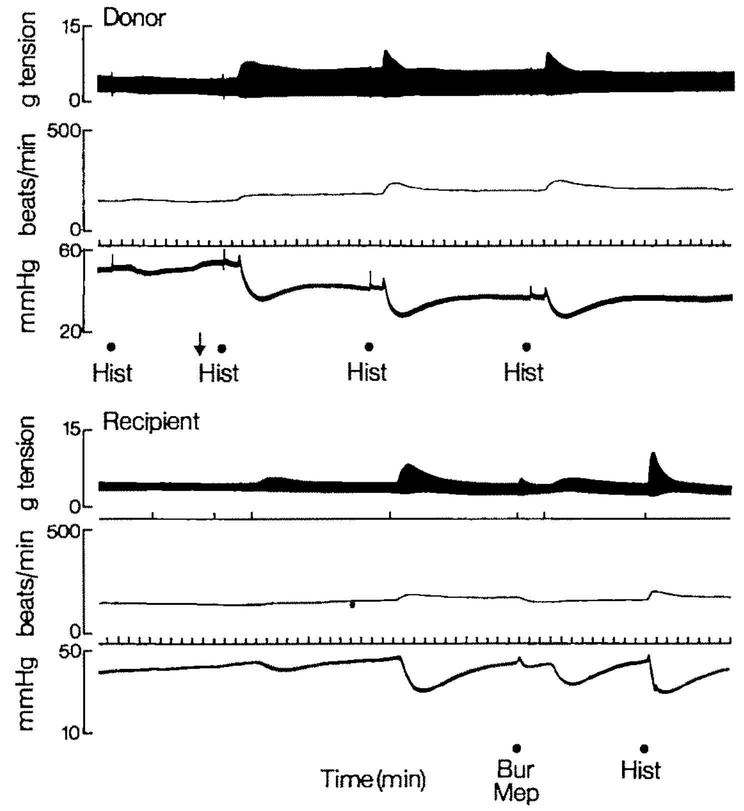


**Figure 5** Continuation of the experiment using the donor and recipient guinea-pig isolated perfused hearts shown in Figure 4. The effects of histamine (Hist, 1 µg) on the contractile force (upper records), heart rate (middle records) and coronary perfusion pressure (lower records) of each heart, initially in the presence of burimamide (20 µg/ml) and mepyramine (1 µg/ml) perfusing from the donor heart reservoir are shown. At ↓ perfusion with a drug-free Krebs-bicarbonate solution was started. After recovery of the responses to histamine, the burimamide (Bur, 2 mg) and mepyramine (Mep, 5 µg) were returned to the recipient heart immediately before an injection of histamine to the donor heart.

parameters were attempted the vasodilatation of either heart could not be improved above that obtained with the quoted values.

Figure 6 also demonstrates the responses of the recipient heart that occur when the 3-way tap was turned to change from fresh Krebs-bicarbonate solution to the perfusate leaving the donor heart. The perfusion pressure fell and the tension usually declined. The effect of topping-up the reservoir with fresh Ringer was to produce a transient increase in pressure, and at the end of the experiment on returning completely to fresh Ringer the vasoconstriction was maintained (Figure 6c).

Finally, experiments were performed in hearts perfused with oxygenated Locke Ringer solution (Figure 7). The donor heart was exposed to a period of anoxia by perfusing with ungassed Ringer solution. There was a reduction in the force of contraction with arrhythmias and a large coronary vasodilatation. All three parameters were restored to normal on returning to oxygenated Locke solution. One minute after the onset of coronary vasodilatation in the donor heart there was a corresponding fall in perfusion pressure in the recipient heart but without the tension and rate changes. This returned to normal in parallel with the pressure changes in the donor heart.

## Discussion

The multi-component coronary vascular response to adrenaline has been demonstrated in guinea-pig isolated perfused hearts. The overall effect of adrenaline is recognized as vasodilatation and an increased coronary blood flow (Wégria, 1951; Charlier, 1961; Parratt, 1968) and this was illustrated

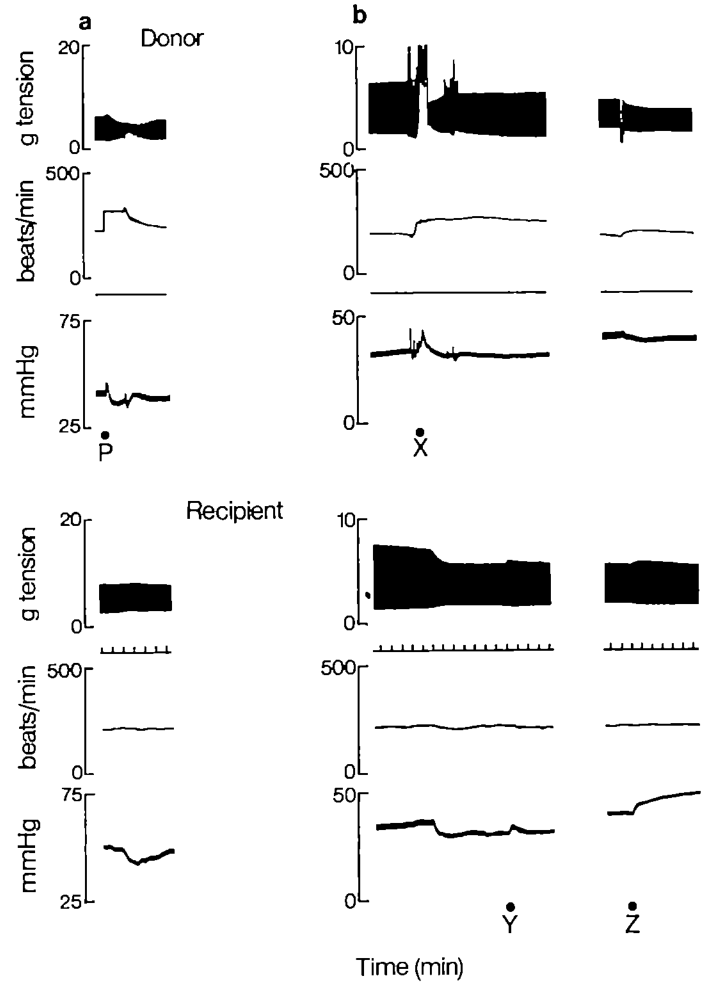


**Figure 6** (a) Donor and recipient isolated perfused (5 ml/min) hearts from guinea-pigs (374 and 328 g respectively). The effects of electrically-paced ventricular tachycardia (P, 5 Hz, 10 V, 5 ms for 2 min) of the donor heart on the contractile force (upper records), heart rate (middle records) and coronary perfusion pressure (lower records) of each heart. (b) Donor and recipient isolated perfused (5 ml/min) hearts from guinea-pigs (431 and 278 g respectively). The effects are shown of connecting the two hearts at the beginning of an experiment and changing from normal Krebs-bicarbonate solution to the perfusate leaving the donor heart (X). At Y the effects of topping-up the recipient heart reservoir with fresh Krebs-bicarbonate solution are shown and at the end of the experiment (Z) the return to fresh Krebs-bicarbonate solution and disconnecting the hearts.

here as a predominant fall in perfusion pressure following an initial vasoconstriction. This vasodilatation is attributed to both a direct stimulation of $\beta$-adrenoceptors in the coronary vasculature (Adam *et al.*, 1970; Parratt & Wadsworth, 1970; Ross & Jorgensen, 1970) and an indirect effect arising from the concomitant increased myocardial activity (Berne, 1964). In the present study stimulation of the donor heart by adrenaline and the accompanying vasodilatation were followed by similar responses in the recipient heart due to the presence of the relatively large dose of adrenaline in the perfusate. When the direct and indirect $\beta$-adrenergic effects of adrenaline were antagonized in the recipient heart by propranolol, stimulation of the donor heart by adrenaline was followed only by the appearance of vasodilator activity in the recipient heart. The time lapse was consistent with the time taken for the perfusate to reach the second heart and the course of the dilatation of both hearts ran approximately in

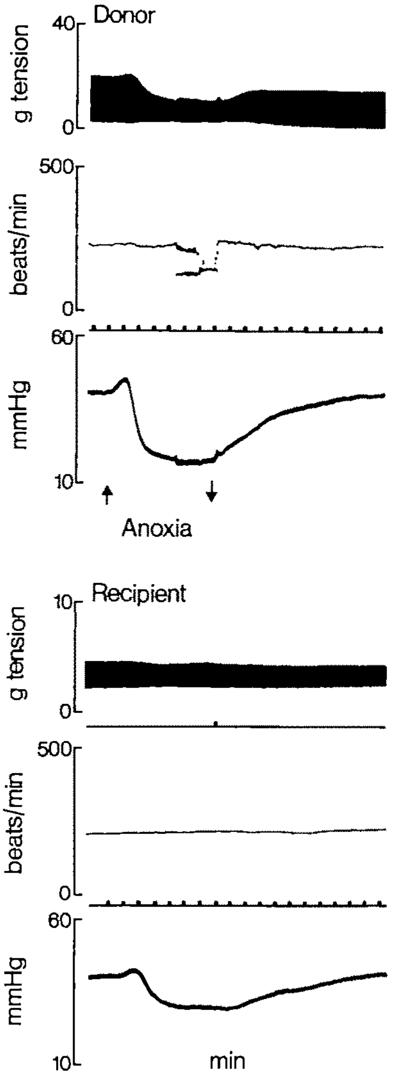


**Figure 7** Donor and recipient isolated hearts from guinea-pigs (518 and 458 g respectively) perfused with Locke Ringer solution (gassed with oxygen) at 5 ml/minute. The effects of a period of perfusion of the donor heart with ungassed Ringer solution (anoxia) between the arrows upon the contractile force (upper records), heart rate (middle records) and coronary perfusion pressure (lower records) of each heart are shown. The perfusate was gassed normally before entry into the recipient heart throughout.

parallel. When the increases in activity of the donor heart were prevented by propranolol then the anticipated vasodilatation no longer occurred. In the case of adrenaline only a rise in pressure remained in both hearts which was attributed to residual direct vasoconstriction of the coronary vessels mediated via $\alpha$-adrenoceptors (Saito, 1959; Kaverina, 1965; Parratt, 1967; Broadley, 1970).

The experiments were repeated with isoprenaline whose direct vascular effects are solely vasodilator. However, the response of the donor heart was biphasic consisting of an initial constriction due to extravascular compression arising from the myocardial effects (Melville & Lu, 1950; Douglas *et al.*, 1960), followed by the characteristic vasodilatation. When the $\beta$-adrenoceptors in the recipient heart were blocked by propranolol this vasodilatation was followed by a vasodilatation in the recipient heart. It is therefore concluded that both adrenaline and isoprenaline, by increasing the rate and force of contraction of the donor heart, cause the release of a vasodilator metabolite into the perfusate.

Increases in myocardial activity were next induced by histamine which, like the catecholamines, produced a multiple coronary vascular response the predominant phase of which was a fall in perfusion pressure. Histamine receptors in the recipient heart were antagonized to prevent their stimulation by histamine carried over in the perfusate. The myocardial effects are mediated via $H_2$-receptors (Black *et al.*, 1972; McNeill & Verma, 1974; Levi *et al.*, 1975) although recent findings suggest that this might only apply to the positive chronotropic responses (Reinhardt, Wagner & Schümann, 1974; Steinberg & Holland, 1975). Nevertheless, both the positive inotropic and chronotropic responses of the recipient heart were abolished here by the $H_2$-antagonist burimamide. The direct constriction and dilatation of coronary vessels mediated via $H_1$-receptors (Broadley, 1975a) were antagonized by mepyramine. Histamine-induced stimulation and vasodilatation of the donor heart were then followed by the appearance of vasodilator activity in the recipient heart.

Thus an increased activity in the donor heart to catecholamines and histamine not only produced a predominant coronary vasodilatation of that heart but has clearly been shown to yield vasodilator activity in the recipient heart on the arrival of the perfusate. It would appear to be due to the presence of a substance in the perfusate released by the increased activity and not by the drugs themselves since abolition of the myocardial response by the appropriate antagonist also prevented the vasodilatation in both hearts. If the release were drug-induced then it would be mediated by identical receptors for the myocardial effects. To eliminate this possibility, experiments were performed in which increases in activity were produced without drugs, namely by electrical pacing. This procedure only increased the rate of contraction, unlike the catecholamines and histamine which increased both rate and tension. In fact the tension usually declined. Although a range of stimulation parameters was attempted, poor vasodilator responses of the donor heart were generally encountered. This was presumably due to an increased resistance from extravascular compression, known to accompany ventricular tachycardia (Anrep & Häusler, 1929; Corday, Gold, DeVera, Williams & Fields, 1959), which opposed the vasodilatation due to the increased activity of the heart. However, the tachycardia of the donor heart was followed by a more prominent vasodilatation in the recipient heart. Electrically-induced tachycardia was therefore also associated with the release of a vasodilator metabolite, although histamine and the catecholamines appeared to be superior in stimulation of the heart and therefore in producing the vasodilator activity. Generally the degree of vasodilatation of both hearts was related to the size of the positive inotropic and chronotropic responses of the donor heart.

The question now arises as to the identity of the metabolite released by the hyperactive heart and whether it is common for all forms of myocardial stimulation. The metabolic link between an increased activity and vasodilatation has been made by several authors (Berne, 1964; Haddy & Scott, 1968). However the presence of a coronary vasodilator metabolite has not previously been pharmacologically demonstrated. Early suggestions as to the nature of the substance included potassium ions (Driscol & Berne, 1957), lactic acid (Mohme-Lundholm, 1957) and bradykinin (Lochner & Parratt, 1966). More recently Berne and his colleagues have proposed (Berne, 1964) that adenosine, which has long been known to possess vasodilator activity (Drury & Szent-Gyorgyi, 1929), is released by the hyperactivity due to catecholamines. They have indeed measured an increased output of this nucleoside from the heart by adrenaline (Katori & Berne, 1966).

Prostaglandins have also been implicated in the regulation of coronary blood flow. Coronary vasodilatation of isolated hearts following adrenaline or electrically-induced tachycardia has been potentiated by the prostaglandin synthetase inhibitor indomethacin (Talesnik & Sunahara, 1973), leading to the suggestion that the prostaglandins exert a suppressant effect upon the vasodilatation. In contrast, prostaglandin $E_2$ has been shown to have coronary vasodilator activity (Krebs & Schrör, 1975) and it has been proposed as the mediator of the coronary vasodilatation that accompanies anoxia (Kent, Alexander, Pisano, Keiser & Cooper, 1973; Wennmalm, Pham-Huu-Chanh & Junstad, 1974). There is a continuous release of prostaglandins by the spontaneously beating heart which is increased following anoxia (Block, Feinberg, Herbaczynska-Cedro & Vane, 1975). However, the visodilatation was not attributed to prostaglandin release since it was not modified by indomethacin, although the release was inhibited.

Anoxia has also been shown to release adenosine (Katori & Berne, 1966), which supports the suggestion that a local anoxia may serve as an intermediate in the predominant vasodilator response to catecholamines due to the increased myocardial activity (Berne, 1958, 1964). Pacing-induced tachycardia has also been shown to produce anoxia (Klarwein, Kako, Chrysohou & Bing, 1961). In the present study, anoxia of the donor heart produced a powerful vasodilatation in that heart which was followed by a similar vasodilatation as the perfusate reached the recipient heart. This could not be a direct effect of the anoxia since the perfusate was reoxygenated before entering the recipient heart. It is therefore concluded that anoxia releases a vasodilator metabolite into the perfusate. Whether this is the same as that released by the catecholamines, histamine and electrical pacing cannot be determined from the present study. However, it is worth speculating that this is a common metabolite whose release is triggered by a local relative anoxia occurring during the myocardial hyperactivity. Prostaglandins or adenosine would appear to be likely candidates and further experiments may clarify their role in the response(s). It has been pointed out however, that prostaglandin release requires adequate oxygen levels, and one would only expect their appearance in the perfusate *after* the period of anoxia (Block *et al.,* 1975). In the present work the vasodilatation in both the donor and recipient hearts occurred while the donor heart was still exposed to the hypoxic Ringer solution or increased activity and this might exclude prostaglandins as possible mediators.

The presence of a vasodilator metabolite has been clearly demonstrated pharmacologically in the perfusate leaving a heart which has undergone a period of increased activity. So far it has been assumed that the fall in perfusion pressure when the perfusate arrives at the recipient heart is due to the presence of a released substance. Alternatively, one could argue that the increased activity of the donor heart merely removes or utilizes one or more constituents of the Ringer solution and that its absence leads to a fall in perfusion pressure. However this possibility can confidently be discounted on the following grounds. It is unlikely that a local change in Ringer composition could occur to such an extent as to affect the donor heart for well after the activity has returned to normal and then to be maintained during transit from donor to recipient heart. Furthermore, in the case of anoxia there is no hyperactivity and this must be a release phenomenon.

It is clear that the substance or substances are probably released from the contracting heart continuously since vasodilatation in the recipient heart occurred immediately the perfusate was taken to this heart. This persisted throughout the experiment and on returning to fresh Ringer solution the perfusion pressure rose again. This resting level of metabolite output is therefore raised by an increased cardiac activity due to drugs or electrical pacing leading to further vasodilatation. The amounts released are presumably very small, yet there is apparently no tachyphylaxis since the vasodilator responses were readily repeatable over experiments of long duration.

This proposed hyperactivity-anoxia-metabolite system for coronary vasodilatation may well be the basis of the autoregulation of the coronary flow that provides the circulatory need of the active myocardium. It now remains to adapt the procedure described here to aid the clarification of the nature of the metabolite and to determine whether a common substance is involved in the procedures used here to induce its release.

I am grateful to Dr R.W. Brimblecome of SK & F for the generous gift of burimamide, to Ward Blenkinsop for the (–)-isoprenaline and to ICI for the propranolol.

**References**

ADAM, K.R., BOYLES, S. & SCHOLFIELD, P.C. (1970). Cardio-selective $\beta$-adrenoceptor blockade and the coronary circulation. *Br. J. Pharmac.*, **40**, 534–536.

ANREP, G.V. & HÄUSLER, H. (1929). The coronary circulation. II. The effect of changes in temperature and of heart rate. *J. Physiol., Lond.*, **67**, 299–314.

BAYER, B.L., MENTZ, P. & FÖRSTER, W. (1972). Über spezifische Unterschiede zwischen den adrenergen $\beta$-Rezeptoren an Hertz und Koronarien. *Arch. int. Pharmacodyn*, **200**, 341–347.

BERNE, R.M. (1958). Effect of epinephrine and norepinephrine on coronary circulation. *Circulation Res.*, **6**, 644–655.

BERNE, R.M. (1964). Regulation of coronary blood flow. *Physiol. Rev.*, **44**, 1–29.

BLACK, J.W., DUNCAN, W.A.M., DURANT, C.J., GANELLIN, C.R. & PARSONS, E.M. (1972). Definition of antagonism of histamine $H_2$-receptors. *Nature, Lond.*, **236**, 385–390.

BLOCK, A.J., FEINBERG, H., HERBACZYNSKA-CEDRO, K. & VANE, J.R. (1975). Anoxia-induced release of prostaglandins in rabbit isolated hearts. *Circulation Res.*, **36**, 34–42.

BROADLEY, K.J. (1970). An analysis of the coronary vascular responses to catecholamines, using a modified Langendorff heart preparation. *Br. J. Pharmac.*, **40**, 617–629.

BROADLEY, K.J. (1975a). The role of $H_1$ and $H_2$-receptors in the coronary vascular response to histamine of isolated perfused hearts of guinea-pigs and rabbits. *Br. J. Pharmac.*, **54**, 511–521.

BROADLEY, K.J. (1975b). A procedure to demonstrate the release of a vasoactive metabolite by catecholamines from perfused guinea-pig hearts. *Br. J. Pharmac.*, **54**, 223–224P.

CHARLIER, R. (1961). *Coronary Vasodilators*. Oxford: Pergamon Press.

CORDAY, E., GOLD, H., DEVERA, L.B., WILLIAMS, J.H. & FIELDS, J. (1959) Effect of the cardiac arrythmias on the coronary circulation. *Ann. intern. Med.*, **50**, 535–553.

DEMPSEY, P.J. & COOPER, T. (1972). Pharmacology of the coronary circulation. *Ann. Rev. Pharmac.*, **12**, 99–110.

DOUGLAS, R.C., ARMENGOL, V. & TALESNIK, J. (1960). Influence of cardiac activity on coronary flow control. *Acta physiol. latinoam.*, **10**, 205–216.

DREW, G.M. & LEVY, G.P. (1972). Characterization of the coronary vascular $\beta$-adrenoceptors in the pig. *Br. J. Pharmac.*, **46**, 348–350.

DRISCOL, T.E. & BERNE, R.M. (1957). Role of potassium in regulation of coronary blood flow. *Proc. Soc. exp. Biol. Med.*, **96**, 505–508.

DRURY, A.N. & SZENT-GYORGYI, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J. Physiol., Lond.*, **68**, 213–237.

ERCAN, Z.S., BÖKESOY, T.A. & TÜRKER, R.K. (1974). A study of the histamine $H_2$-receptors in heart muscle and coronary vessels. *Eur. J. Pharmac.*, **27**, 259–262.

GARCIA-RAMOS, J., ALANIS, J. & LUCO, J. (1950). Estudios sobre la circulation coronaria. II. Las acciones del vago y del simpatico. *Archos Inst. Cardiol. Méx.*, **20**, 534–550.

GREEN, H.D., WÉGRIA, R. & BOYER, N.H. (1942). Effect of epinephrine and pitressin on the coronary artery inflow in anaesthetized dogs. *J. Pharmac. exp. Ther.*, **76**, 378–391.

HADDY, F.J. & SCOTT, J.B. (1968). Metabolically linked vasoactive chemicals in local regulation of blood flow. *Physiol. Rev.*, **48**, 688–707.

HARDIN, R.A., SCOTT, J.B. & HADDY, F.J. (1961). Effects of epinephrine and norepinephrine on coronary vascular resistance in dogs. *Am. J. Physiol.*, **201**, 276–280.

HASHIMOTO, K., SHIGEI, T., IMAI, S., SAITO, Y., YAGO, N., UEI, I. & CLARK, R.E. (1960). Oxygen consumption and coronary vascular tone in the isolated fibrillating dog heart. *Am. J. Physiol.*, **198**, 965–970.

KATORI, M. & BERNE, R.M. (1966). Release of adenosine from anoxic hearts. Relationship to coronary flow. *Circulation Res.*, **19**, 420–425.

KAVERINA, N.V. (1965). *Pharmacology of the Coronary Circulation*. Oxford: Pergamon Press.

KENT, K.M., ALEXANDER, R.W., PISANO, J.J., KEISER, H.R. & COOPER, T. (1973). Prostaglandin dependent coronary vasodilator responses. *Physiologist*, **16**, 361.

KLARWEIN, M., KAKO, K., CHRYSOHOU, A. & BING, R.J. (1961). Effect of atrial and ventricular fibrillation and ventricular tachycardia on carbohydrate metabolism of the heart. *Circulation Res.*, **9**, 819–825.

KREBS, R. & SCHRÖR, K. (1975). Actions of prostaglandin $E_2$ on myocardial mechanics, coronary vascular resistance and oxygen consumption in the guinea-pig isolated heart preparation. *Br. J. Pharmac.*, **55**, 403–408.

LANGENDORFF, O. (1895). Untersuchungen am überlebenden Säugetierherzen. *Pflügers Arch. ges. Physiol.*, **61**, 291–332.

LEVI, R., CAPURRO, N. & LEE, C.-H. (1975). Pharmacological characterization of cardiac histamine receptors: sensitivity to $H_1$- and $H_2$-receptor agonists and antagonists. *Eur. J. Pharmac.*, **30**, 328–335.

LEVI, R. & KUYE, J.O. (1974). Pharmacological characterization of cardiac histamine receptors: sensitivity to $H_1$-receptor antagonists. *Eur. J. Pharmac.*, **27**, 330–338.

LOCHNER, W. & PARRATT, J.R. (1966). A comparison of the effects of locally and systemically administered kinins on coronary blood flow and myocardial metabolism. *Br. J. Pharmac. Chemother.*, **26**, 17–26.

LUCHESSI, B.R. & HODGEMAN, R.J. (1971). Effect of 4-(2-hydroxy-3-isopropylaminopropoxy) acetanilide (AY 21,011) on the myocardial and coronary vascular responses to adrenergic stimulation. *J. Pharmac. exp. Ther.*, **176**, 200–211.

McNEILL, J.H. & VERMA, S.C. (1974). Blockade by burimamide of the effects of histamine and histamine analogs on cardiac contractility, phosphorylase activation and cyclic adenosine monophosphate. *J. Pharmac. exp. Ther.*, **188**, 180–188.

MARK, A.L., ABBOUD, F.M., SCHMID, P.G., HEISTAD, D.D. & MAYER, H.E. (1972). Differences in direct effects of adrenergic stimuli on coronary, cutaneous and muscular vessels. *J. clin. Invest.*, **51**, 279–287.

MELVILLE, K.I. & LU, F.C. (1950). Effects of epinephrine, aminophylline, nitroglycerine and papaverine on coronary inflow and on heart contractions as recorded concurrently. *J. Pharmac. exp. Ther.*, **99**, 286–303.

MOHME-LUNDHOLM, E. (1957). Mechanism of the relaxing effect of adrenaline on bovine coronary vessels. *Acta physiol. scand.*, **38**, 255–264.

PARRATT, J.R. (1967). Adrenergic receptors in the coronary circulation. *Am. Heart J.*, **73**, 137–140.

PARRATT, J.R. (1968). Pharmacological aspects of the coronary circulation. In *Progress in Medicinal Chemistry*, ed. Ellis, G.P. & West, G.B., vol. 6, pp. 11–66. London: Butterworth & Co. Ltd.

PARRATT, J.R. & WADSWORTH, R.M. (1970). The effect of "selective" $\beta$-adrenoceptor blocking drugs on the myocardial circulation. *Br. J. Pharmac.*, **39**, 296–308.

PARRATT, J.R. & WADSWORTH, R.M. (1972). The effects of dipyridamole on the myocardial vasodilator actions of noradrenaline, isoprenaline and adenosine. *Br. J. Pharmac.*, **46**, 585–593.

REINHARDT, D., WAGNER, J. & SCHÜMANN, H.J. (1974). Differentiation of $H_1$- and $H_2$-receptors mediating positive chrono- and inotropic responses to histamine on atrial preparations of the guinea-pig. *Agents and Actions*, **4**, 217–221.

ROSS, G. (1974). Myocardial and coronary vascular effects of salbutamol. *Cardiovasc. Res.*, **8**, 1–7.

ROSS, G. & JORGENSEN, C.R. (1970). Effects of a cardio-selective beta-adrenergic blocking agent on the heart and coronary circulation. *Cardiovasc. Res.*, **4**, 148–153.

SAITO, H. (1959). Effects of adrenaline and noradrenaline on the coronary outflow of the isolated rabbit heart. *Bull. Osaka med.* Sch., **5**, 15–33.

SIEGAL, J.H., GILMORE, J.P. & SARNOFF, J.J. (1961). Myocardial extraction and production of catecholamines. *Circulation Res.*, **9**, 1336–1350.

STEINBERG, M. & HOLLAND, D.R. (1975). Separate receptors mediating the positive inotropic and chronotropic effects of histamine in guinea-pig atria. *Eur. J. Pharmac.*, **34**, 95–104.

TALESNIK, J. & SUNAHARA, F.A. (1973). Enhancement of metabolic coronary dilatation by aspirin-like substances by suppression of prostaglandin feed-back control? *Nature, Lond.*, **224**, 351–353.

WÉGRIA, R. (1951). Pharmacology of the coronary circulation. *Pharmac. Rev.*, **3**, 197–246.

WENNMALM, Å., PHAM-HUU-CHANH & JUNSTAD, M. (1974). Hypoxia causes prostaglandin release from perfused rabbit hearts. *Acta physiol. scand.*, **91**, 133–135.

*(Received January 30, 1976.
Revised March 16, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 101–107

# MORPHINE–NALOXONE INTERACTION IN THE CENTRAL CHOLINERGIC SYSTEM: THE INFLUENCE OF SUBCORTICAL LESIONING AND ELECTRICAL STIMULATION

K. JHAMANDAS & M. SUTAK
Department of Pharmacology, Faculty of Medicine, Queen's University, Kingston, Ontario, Canada K7L 3N6

1 The opiate antagonist naloxone, injected or topically applied to the cerebral cortex, had no significant effect on the spontaneous output of cortical acetylcholine (ACh) in rats.

2 Morphine (2.5 mg/kg) administered intravenously inhibited the release of cortical ACh. A subsequent injection of naloxone rapidly reversed morphine-induced inhibition, and produced a sustained increase in the release of ACh. Topical application of naloxone solutions, after morphine, produced a slow and weak reversal of its inhibitory action.

3 Destruction of the medial thalamus abolished both the inhibitory effects of morphine on the cortical ACh release, and its antagonism by naloxone administered after the agonist.

4 Injection of naloxone in a low dose (0.1 mg/kg) increased the release of cortical ACh provoked by electrical stimulation of either the medial thalamus or the reticular formation in normal rats. In the morphine-dependent rat, naloxone also facilitated the evoked release and its action was greater than in control animals. The facilitatory effect of naloxone on the cortical release evoked by stimulation of the medial thalamus was greater than its effect on the release evoked by stimulation of the reticular formation in both normal and morphine-dependent rats.

5 Naltrexone, a narcotic antagonist, also facilitated the electrically stimulated release of cortical ACh.

6 It is suggested that (a) morphine and naloxone act at a subcortical site, probably the medial thalamus, to modify the cortical ACh release and that (b) naloxone may facilitate the electrically-induced release of ACh in the CNS by antagonizing the effect of the endogenous morphine-like factor, enkephalin.

## Introduction

The development of a precipitated abstinence syndrome in opiate-dependent animals is characterized by an abnormal increase in the cholinergic activity. It has been suggested that a sudden increase in the release of acetylcholine (ACh) in the periphery and the central nervous system (CNS) could contribute to the excessive cholinergic activity associated with the abstinence phenomenon (Paton, 1963; Crossland, 1970). Studies reported from this and other laboratories show that the opiate antagonist naloxone, when administered to chronically morphine-treated animals, enhances the output of ACh in the cerebral cortex (Jhamandas & Sutak, 1974; Labrecque & Domino, 1974; Mullins & Phillis, 1974). Morphine itself can inhibit the release of ACh in the CNS and this effect can be antagonized by naloxone administered before or after morphine treatment (Jhamandas, Phillis & Pinsky, 1971; Matthews, Labrecque & Domino, 1973; Yaksh & Yamamura, 1975). Furthermore, the development of some signs of abstinence precipitated by naloxone, in morphine-dependent rats, is suppressed by pretreatment with ACh antagonists (Collier, Francis & Schneider, 1972; Jhamandas & Dickinson, 1973; Jhamandas, Sutak & Bell, 1973). These observations lend support to the suggestion that the ACh releasing system in the CNS could play a significant role in the development of the opiate abstinence syndrome precipitated by naloxone or related antagonists. However, the site(s) in the central cholinergic system at which naloxone, or morphine, may be acting to modify the release of ACh remain unknown.

Recently some investigators have suggested that the medial thalamus is an important brain site involved in the development of tolerance to the EEG effects of morphine (Teitelbaum, Catravas & McFarland, 1974) and the naloxone-precipitated abstinence syndrome in the rat (Wei, Loh & Way, 1973a and b). It has also been demonstrated that there is a high degree of opiate receptor binding in this area (Kuhar, Pert & Snyder,

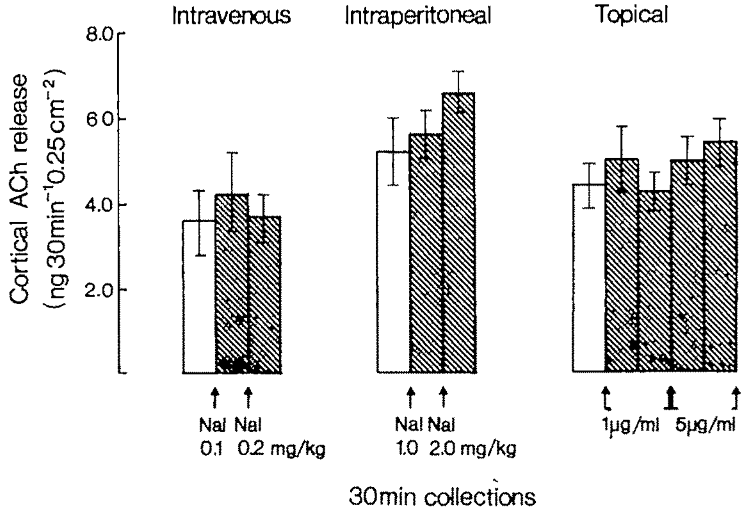


**Figure 1** The effect of injected and topically applied naloxone (Nal) on the output of acetylcholine (ACh) from the cortical surface of the cerebral cortex. Naloxone was injected or applied at the points indicated by arrows. Each column represents the mean output in six experiments with s.e. mean. Open columns indicate release preceding the drug injections.

1973). The region of the medial thalamus is an integral part of the ascending reticular cholinergic system (Shute & Lewis, 1967) which projects diffusely to the cortex and releases ACh in this region. These observations make it attractive to consider that the action of naloxone in a morphine-treated animal and possibly also the effect of morphine on the cortical release of ACh, may be exerted at this region. If this idea were correct, lesioning of the medial thalamus should abolish the effects of naloxone and morphine on the release of cortical ACh. The objective of the present study was to see if this were the case.

In previous tests, naloxone has been shown to have no significant action of its own on the spontaneous output of central ACh. However, in a recent report, Waterfield & Kosterlitz (1975) demonstrated that naloxone, and other opiate antagonists, increased the electrically stimulated release of ACh in the guinea-pig myenteric plexus-longitudinal muscle preparation. A stereospecificity of this effect was also demonstrated, and it was suggested by these investigators that the opiate antagonists may be enhancing the stimulated release by antagonizing the action of an endogenous morphine-like substance, enkephalin, which is present in this tissue (Hughes, Smith, Morgan & Fothergill, 1975). The second objective of our study was to determine whether naloxone would increase the central release of ACh evoked by electrical stimulation in the CNS as it does in the periphery.

## Methods

### *Release of cortical acetylcholine*

All experiments described here were carried out on Sprague–Dawley rats weighing 250–350 g which were lightly anaesthetized with pentobarbitone (30 mg/kg) and urethane (400 mg/kg) mixture. The release of cortical ACh was investigated in the presence of neostigmine (50 μg/ml) and atropine (0.5 μg/ml) using the cup technique of MacIntosh & Oborin (1953). Experimental details of the procedure, as employed in the rat, have been described fully in a previous paper (Jhamandas & Sutak, 1974).

### *Lesioning and stimulation*

Thermal lesions were placed bilaterally in the medial thalamus with a radio frequency lesion generator (David Kopf Instruments, Model RFG-4) using co-ordinates (AP3.4, L ± 1.0, HV–1.0) according to the rat stereotaxic atlas compiled by Pellegrino & Cushman (1967). Electrical stimulation was applied to the same area, and also to the reticular formation (AP 1.6, L 2.0, HV–2.5), by a concentric electrode 0.5 μm in diameter. Stimuli of 0.3 ms duration, rate of 10 Hz for 1 s, repeated every 10 s, were delivered through a Grass Stimulator (Model S-88). Electrode positions and sites of lesions were verified histologically.

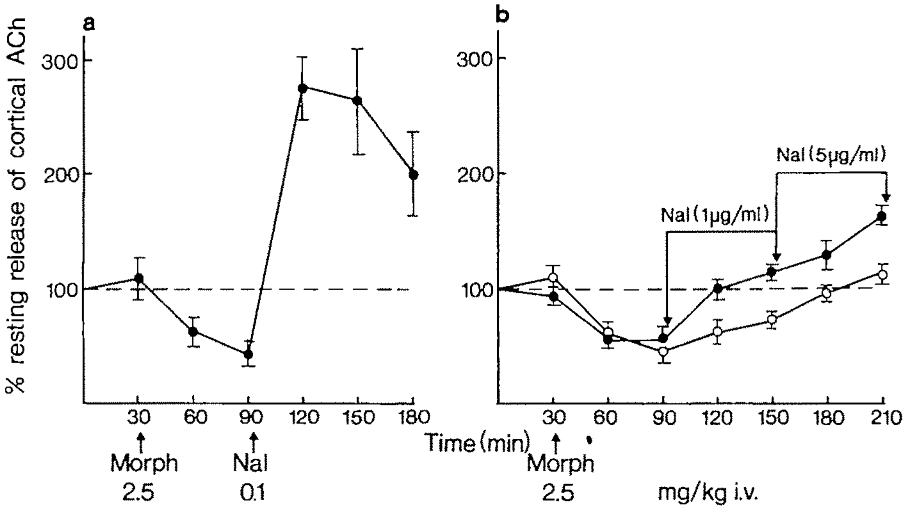


**Figure 2** The reversal of morphine-induced (Morph) inhibition of acetylcholine (ACh) release by the antagonist naloxone (Nal) after (a) its injection or (b) topical application to the left cortex. In both (a) and (b) the mean results of four experiments are shown; vertical lines show s.e. mean. The average resting release in the first two collections, which is represented as 100% was 3.4 ± 0.4 and 4.1 ± 0.5 ng 30 $min^{-1}$ 0.25 $cm^{-2}$ in (a) and (b) respectively. (O) = Control.

### *Assay of acetylcholine*

The solutions collected from the cerebral cortex were assayed biologically on the hearts of the clam *Mercenaria mercenaria* as described previously (Jhamandas *et al.*, 1971).

### *Drugs and treatment*

Drugs used were atropine sulphate, naloxone hydrochloride, naltrexone hydrochloride, neostigmine bromide, pentobarbitone sodium and urethane. All drug solutions, except those applied topically, were prepared by dissolving the drugs in 0.9% w/v NaCl solution (saline). Weights of drugs refer to the salts. Chronic morphine injections were administered to rats as described in a previous paper (Jhamandas & Sutak, 1974).

## Results

### *Effects of naloxone and morphine on the spontaneous release of acetylcholine*

The histograms in Figure 1 show results of experiments in which the effects of naloxone alone were investigated on the spontaneous release of cortical ACh following its injection or topical application. Injections of the drug in doses 0.1–0.2 mg/kg intravenously, and 1.0–2.0 mg/kg intraperitoneally had no significant effect on this release. Similarly an application of two naloxone solutions (1 and 5 μg/ml) to the cortex, each for a total of 60 min, did not affect the resting output of ACh. In separate experiments, the effects of injected and locally applied naloxone were investigated following pretreatment with morphine (2.5 mg/kg i.v.). Morphine consistently reduced the output of ACh to about 50% of the predrug value (Figure 2). A subsequent injection of naloxone (0.1 mg/kg) not only induced a complete reversal of the morphine effect but caused the output of ACh to exceed the value of resting release by nearly 200% (Figure 2a). This effect was sustained and it did not begin to decline towards the resting value until the third post-naloxone collection period. It was reasoned that if naloxone was acting at the cortical level to reverse morphine-induced depression of release, then its local application at this site should produce a comparable degree of antagonism. In the next experiment, results of which are shown in Figure 2b, two solutions of naloxone were applied to the cortex unilaterally after depression of the release of ACh with intravenously injected morphine. Although a reversal of this depression by naloxone was discernible, as indicated by a more rapid recovery of release in the treated side, this antagonism was sluggish when compared with the large and rapid effect of the injected drug in the previous tests. Despite a prolonged application period, the release of ACh following topical naloxone was never as high as that observed after its injection. These results suggested that the

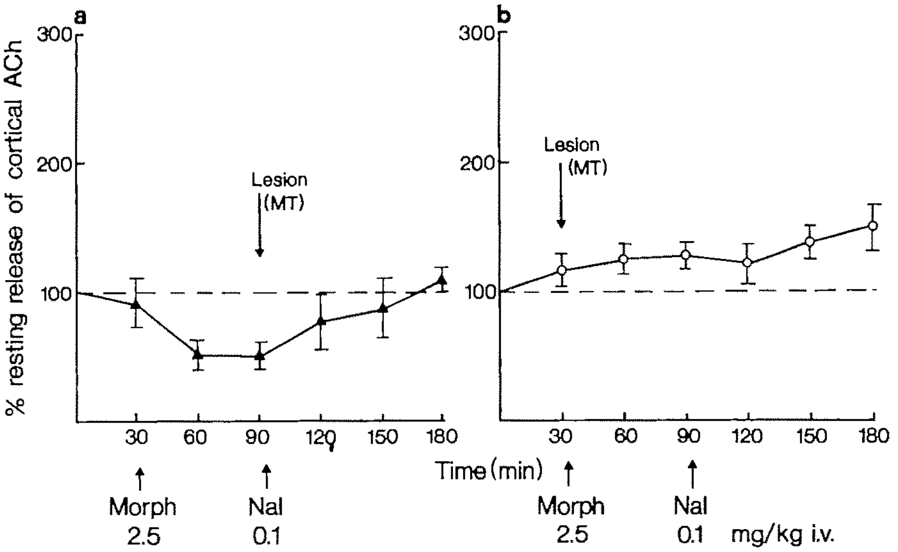


**Figure 3** The effect of lesioning medial thalamus (MT) on (a) the antagonism of morphine effect on acetylcholine (ACh) release by naloxone and (b) the inhibitory effect of morphine on ACh release. Naloxone (Nal) and morphine (Morph) were injected 5 min after making the lesion in (a) and (b), respectively. Mean of 4 experiments is shown in (a) and of 6 experiments in (b); vertical lines show s.e. mean. The average resting release (100%) values were 3.8±0.7 and 2.6±0.2 ng 30 min$^{-1}$ 0.25 cm$^{-2}$.

antagonist may have been diffusing slowly from the cortex to a subcortical site and subsequently acting there to reverse the action of the agonist.

### *Naloxone and morphine effect on acetylcholine release after lesion placement*

The possibility that naloxone may be antagonizing the action of morphine on the cortical ACh release by acting at a subcortical site was investigated in experiments where the medial thalamus was lesioned before naloxone injection. The effect of morphine itself was also investigated in similar tests. Results of the lesion experiments are shown in Figure 3a and b. In preliminary tests it was established that the introduction of the lesion probes, or the electrodes into the brain, had no significant action on the basal output of cortical ACh. As shown in Figure 3a, a morphine injection (2.5 mg/kg) produced the expected inhibition of the cortical ACh release. When an inhibition had occurred, the medial thalamus was lesioned bilaterally and, 5 min later, an injection of naloxone (0.1 mg/kg) was administered to the animal. Naloxone did not now produce the large increase in the cortical release of ACh as it had done in the previous experiments shown in Figure 2a. The release of ACh after morphine injection and lesioning reached the control level in the next 30 min period following a lesion. This post-morphine recovery of the release of ACh to control levels was apparently more rapid than the recovery from the same dose of morphine in earlier tests which did not involve lesioning (Figure 2b, control side). This observation suggested that lesioning may have destroyed the site(s) at which morphine was exerting its inhibitory effect, and thereby terminated the agonist action. This possibility was tested in other experiments where lesions in the medial thalamus were placed before an injection of morphine (2.5 mg/kg). As shown in Figure 3b the agonist now failed to depress the cortical ACh release. A post-morphine injection of naloxone also did not modify the release.

### *Effects of naloxone on electrically stimulated acetylcholine release*

The effects of a low dose of naloxone (0.1 mg/kg) were investigated on the cortical output of ACh evoked by electrical stimulation delivered to the medial thalamus in some tests, and the mesencephalic reticular formation in others. Experiments were carried out in normal rats, and in animals that had been chronically treated with repeated injections of morphine. Preliminary tests in the normal animals indicated that two successive periods of electrical stimulation, delivered at 10 Hz to the same region and when separated by 60–90 min, consistently provoked similar increments in the output of cortical ACh over its pre-stimulus value. This observation allowed the investigation of drug effect on the evoked output in the same animal by using one stimulation period as the

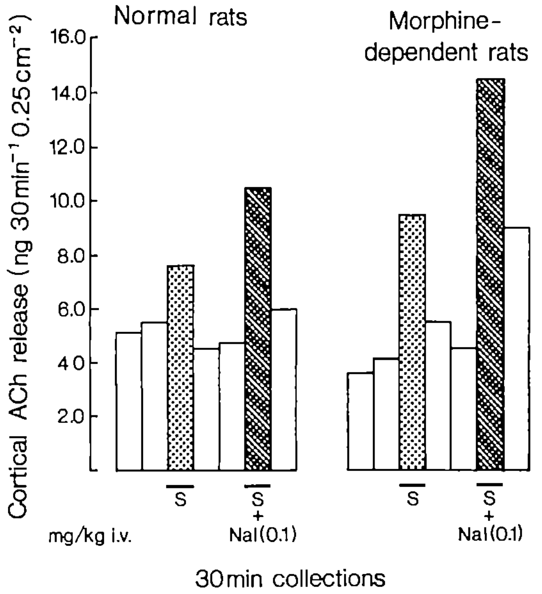


**Figure 4** Effects of electrical stimulation of the medial thalamus on the release of cortical acetylcholine (ACh) before and after a naloxone (Nal) injection in a normal rat and a morphine-dependent rat (single experiments). Stimulation (S) was applied, during the periods indicated by the bar, immediately following the drug injection.

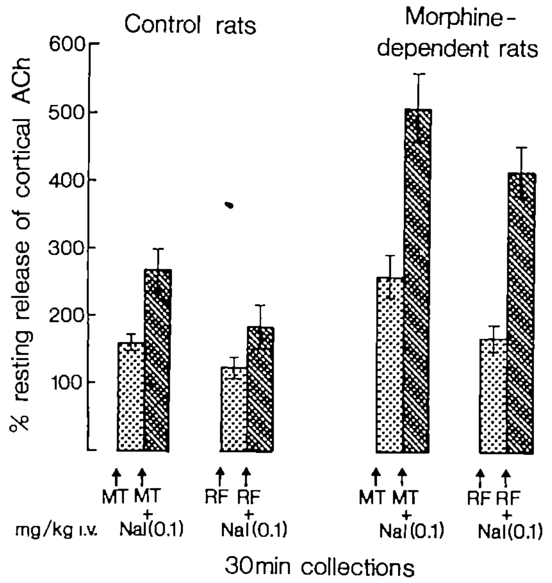


**Figure 5** Effect of electrical stimulation of the medial thalamus (MT) or the reticular formation (RF) before and after naloxone injection in normal and morphine-dependent rats. The results obtained from four to six experiments, of the type shown in Figure 4, have been normalized by expressing the stimulated release as a percentage of the pre-stimulus resting release. In experiments involving MT and RF stimulation the average values, expressed as ng 30 min$^{-1}$ 0.25 cm$^{-2}$, for resting release were: control rats, 4.7±0.3 ($n=6$) and 3.7±0.9 ($n=4$), respectively; morphine-dependent rats, 3.0±0.2 ($n=4$) and 3.3±0.8 ($n=4$).

control, and delivering the other in combination with the drug under investigation.

The results of representative tests with naloxone in normal and morphine-dependent animals are shown in Figure 4, while the combined results of several tests are shown in Figure 5. As shown in Figure 4 (left histogram) the electrical stimulation of the medial thalamus alone in the normal rat increased the cortical ACh output by 50% over the pre-stimulus resting level. When the stimulation was delivered immediately after naloxone the release of ACh rose to about 120% over the resting value, indicating a clear facilitation of the electrically evoked release by the antagonist. The low dose of naloxone used here was without effect on the spontaneous release as observed earlier (Figure 1). In a comparative test on the morphine-dependent animal, also shown in Figure 4 (right histogram), the stimulation alone increased the release by 150% and its combination with naloxone treatment caused the output to rise to nearly 300% over the resting value. This indicated that pretreatment with morphine greatly intensified the stimulatory effect of naloxone on the electrically induced release of ACh from the cortex. The morphine-dependent animals, although under the influence of the anaesthetic, in these tests exhibited mild classical signs of opiate withdrawal soon after receiving naloxone. These signs did not appear in the normal rats but piloerection and salivation was often observed in these animals.

The results of several experiments in which naloxone effects were tested on the electrical stimulation of either the medial thalamus, or the reticular formation are shown in Figure 5. As shown here, naloxone facilitated the electrically evoked release when both subcortical sites were stimulated. However, its action on the evoked release following medial thalamus stimulation were greater in magnitude, in both normal and morphine-treated animals, suggesting a possibly greater sensitivity of this site to the antagonist's action.

In order to investigate whether the actions of naloxone were shared by the other opiate antagonists its effects were compared with those of a related agent, naltrexone. Initial tests with a low dose of naltrexone (0.1 mg/kg) indicated a lack of significant effect on the spontaneous release when administered by itself, and a rapid reversal of morphine effect when administered after the agonist. The effects of naltrexone on the release of ACh evoked by the stimulation of medial

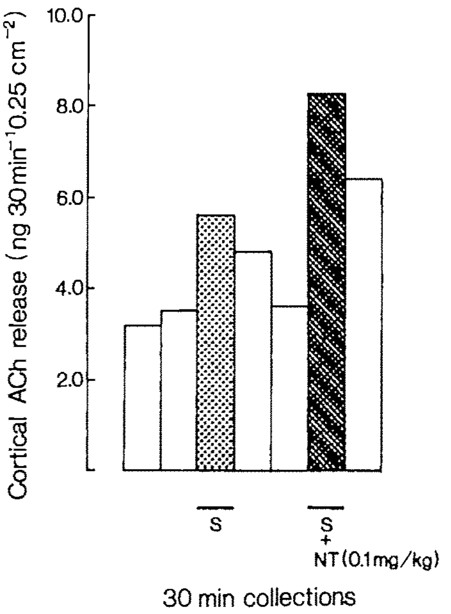


**Figure 6** Effect of naltrexone (NT) on the release of cortical acetylcholine (ACh) evoked by the electrical stimulation of the medial thalamus in a normal rat (single experiment). Stimulation (S) was applied during the period indicated by the bars, immediately following the drug injection.

thalamus are shown in Figure 6; like naloxone, it markedly increased the stimulated output of ACh in three of the five experiments on normal animals. Its effect in the morphine-dependent animals was not tested.

## Discussion

The inhibitory effects of morphine on the release of cortical ACh could result from its action at the cholinergic nerve terminals in the cortex, or at some subcortical site in the ascending system of cholinergic neurones projecting to the cortex. Evidence from previous investigations with morphine favours the latter possibility. These studies have shown that application of morphine solutions to one side of the cortex depresses the release of ACh on both sides of the cortex, possibly by diffusion of the drug to a subcortical site (Jhamandas, Pinsky & Phillis, 1970). Szerb (1974) has reported that the release of labelled ACh from isolated slices of the cortex and other regions was not inhibited in the presence of morphine. In a recent study of two narcotic agonists, methadone and levorphanol, and their isomers, it was found that a clear stereospecificity of inhibitory action of the cortical ACh release could be observed *in vivo* but not *in vitro* (Jhamandas, Hron & Sutak, 1975). The experiments described here, in which lesions placed in a lower level resulted in a loss of morphine effect, provide further evidence for a subcortical site of action. The fact that both the depressant effect of morphine on release of ACh and its antagonism by naloxone were abolished following lesioning of the medial thalamus suggests that this region could be an important site for an interaction between the opiate agonist and its antagonist occurring in the ascending cholinergic system. The slow antagonism of injected morphine by cortically applied naloxone observed here could have resulted from a slow diffusion of the antagonist to this region. Since the medial thalamus is interposed between the brainstem reticular formation, where the outflow of the ascending cholinergic system originates, and the cerebral cortex, where ACh is released, the possibility of an even lower site of action cannot be excluded on the basis of the present experiments. In this context, Garau, Mulas & Pepeu (1975) have reported that a loss of morphine action on the cortical ACh release results following lesions of the raphe nuclei in the brainstem.

Although naloxone has been shown to have no significant action on the spontaneous release, the experiments described here show that it greatly increases the electrically stimulated release of ACh. This finding in the CNS is similar to that of Waterfield & Kosterlitz (1975) in the guinea-pig myenteric plexus, where naloxone and two benzomorphan antagonists were found to enhance the electrically induced release of ACh. Since a stereospecificity of this action with antagonists of the benzomorphan series could be demonstrated, these authors suggested that the opiate antagonists may be enhancing the stimulated release by antagonizing the action of enkephalin which is present in the guinea-pig ileum and inhibits the release of ACh in this preparation (Hughes *et al.*, 1975). Although a stereospecificity of the facilitatory action of antagonists on the evoked release was not investigated in the present tests, the results obtained could be interpreted in similar terms. The facilitation of central ACh release by naloxone may reflect the antagonism of enkephalin or a morphine-like factor (Hughes, 1975; Pasternak, Goodman & Snyder, 1975) which is perhaps released following electrical stimulation. The possibility of the facilitatory effect being simply a non-specific action of naloxone is diminished by the fact that it was (a) apparent at a low dose of the antagonist, (b) intensified by making the animals morphine-dependent and (c) observed with another selective antagonist, naltrexone. It is also apparent that the facilitation of stimulated release was greater following the thalamic stimulation than the reticular stimulation. The reason for this difference is not clear, however, it is attractive to suggest that the difference could be due to a greater release of enkephalin by the thalamic stimulation, and consequently a more effective antagonism by naloxone. These observations suggest that the action of enkephalin on central release of ACh, and its antagonism by naloxone, should be evaluated in future experiments.

This investigation was supported by the Medical Research Council of Canada and the Non-Medical Use of Drugs Directorate, National Health and Welfare, Canada. The gift of naloxone and naltrexone (Endo Laboratories) is gratefully acknowledged. We thank Dr H.W. Kosterlitz for helpful discussion of this work and Dr G.S. Marks for helpful criticism of the manuscript.

**References**

COLLIER, H.O.J., FRANCIS, D.L. & SCHNEIDER, C. (1972). Modification of morphine withdrawal by drug interacting with humoral mechanisms: Some contradictions and their interpretation. *Nature, Lond.*, **237**, 220–223.

CROSSLAND, J. (1970). Acetylcholine and morphine abstinence syndrome. In *Drugs and Cholinergic Mechanisms in the C.N.S.* ed. Heilbronn, E. & Winter, A. pp. 634–645. Stockholm: Försvarets Forkningsanstalt.

GARAU, L., MULAS, M.L. & PEPEU, G. (1975). The influence of raphe lesions on the effect of morphine on nociceptive and cortical ACh output. *Neuropharmac.*, **14**, 259–263.

HUGHES, J. (1975). Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. *Brain Res.*, **88**, 295–308.

HUGHES, J., SMITH, T., MORGAN, B. & FOTHERGILL, L. (1975). Purification and properties of enkephalin—the possible endogenous ligand for the morphine receptor. *Life Sci.*, **16**, 1753–1758.

JHAMANDAS, K. & DICKINSON, G. (1973). Modification of methadone abstinence in mice by acetylcholine antagonists. *Nature, New Biol.*, **245**, 219–220.

JHAMANDAS, K., HRON, V. & SUTAK, M. (1975). Comparative effects of opiate agonists methadone, levorphanol and their isomers on the release of cortical ACh in vivo and in vitro. *Can. J. Pharmac. Physiol.*, **50**, 57–62.

JHAMANDAS, K., PHILLIS, J.W. & PINSKY, C. (1971). Effect of narcotic analgesics and antagonists on the IN VIVO release of acetylcholine from the cerebral cortex of the cat. *Br. J. Pharmac.*, **43**, 53–66.

JHAMANDAS, K., PINSKY, C. & PHILLIS, J.W. (1970). Effect of morphine and its antagonists on release of cerebral acetylcholine. *Nature, Lond.*, **228**, 176–177.

JHAMANDAS, K. & SUTAK, M. (1974). Modification of brain acetylcholine release by morphine and its antagonist in normal and morphine-dependent rats. *Br. J. Pharmac.*, **50**, 57–62.

JHAMANDAS, K., SUTAK, M. & BELL, S. (1973). Modification of precipitated morphine withdrawal syndrome by drugs affecting cholinergic mechanisms. *Eur. J. Pharmac.*, **24**, 296–305.

KUHAR, M.J. PERT, C.N. & SNYDER, S.H. (1973). Regional distribution of opiate receptor binding in monkey and human brain. *Nature, Lond.*, **245**, 447–450.

LABRECQUE, G. & DOMINO, E.F. (1974). Tolerance to and physical dependence on morphine: relation to neocortical acetylcholine release in the cat. *J. Pharmac. exp. Ther.*, **191**, 189–200.

MACINTOSH, F.C. & OBORIN, P.E. (1953). Release of acetylcholine from intact cerebral cortex. *Abstr. XIX int. Physiol. Cong.*, pp. 580–581.

MATTHEWS, J.D., LABRECQUE, G. & DOMINO, E.F. (1973). Effects of morphine, nalorphine and naloxone on neocortical release of acetylcholine in the rat. *Psychopharmacologia*, **29**, 113–120.

MULLINS, W.J. & PHILLIS, J.W. (1974). Acetylcholine release from the brain in unanaesthetized cats following habituation to morphine and during precipitation of abstinence syndrome. *Psychopharmacologia*, **36**, 85–99.

PASTERNAK, G.W., GOODMAN, R. & SNYDER, S.H. (1975). An endogenous morphine-like factor in the brain. *Life Sci.*, **16**, 1765–1769.

PATON, W.D.M. (1963). Cholinergic transmission and acetylcholine output. *Can. J. Biochem. Physiol.*, **41**, 2637–2653.

PELLEGRINO, L.J. & CUSHMAN, A.J. (1967). *A Stereotaxic Atlas of the Rat Brain.* New York: Appleton-Century Crofts.

SHUTE, C.C.D. & LEWIS, P.R. (1967). The ascending cholinergic reticular system: neocortical, olfactory and subcortical connections. *Brain*, **90**, 497–519.

SZERB, J.C. (1974). Lack of effect of morphine in reducing the release of labelled acetylcholine from brain slices stimulated electrically. *Eur. J. Pharmac.*, **29**, 192–194.

TEITELBAUM, H., CATRAVAS, G.N. & MCFARLAND, W.L. (1974). Reversal of morphine tolerance after medial thalamic lesions in rat. *Science*, **185**, 449–451.

WATERFIELD, A. & KOSTERLITZ, H.W. (1975). Stereospecific increase by narcotic antagonists of evoked acetylcholine output in guinea-pig ileum. *Life Sci.*, **16**, 1787–1792.

WEI, E., LOH, H.H. & WAY, E.L. (1973a). Brain sites of precipitated abstinence in morphine-dependent rats. *J. Pharmac. exp. Ther.*, **185**, 108–115.

WEI, E., LOH, H.H. & WAY, E.L. (1973b). Neuroanatomical correlates of morphine dependence. *Science*, **177**, 616–617.

YAKSH, T.L. & YAMAMURA, H.I. (1975). Blockade by morphine of acetylcholine release from the caudate nucleus in mid-pontine pretrigeminal cat. *Brain Res.*, **83**, 520–524.

*(Received February 3, 1976.*
*Revised March 16, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 109–116

# THE EFFECT OF CATECHOLAMINES ON THE INFLUX OF CALCIUM AND THE DEVELOPMENT OF TENSION IN DENERVATED MOUSE DIAPHRAGM MUSCLE

R.H. EVANS & J.W. SMITH[1]
Department of Pharmacology, The Medical School,
University Walk, Bristol BS8 1TD

**1** The nature of the catecholamine-induced contracture of chronically denervated mouse diaphragm muscle has been investigated and compared with the contractural response evoked by acetylcholine.

**2** The time course of onset of catecholamine-sensitivity in denervated diaphragm muscles was similar to the development of acetylcholine sensitivity. However, catecholamine contractures were absent in tissues denervated for periods longer than 90 days whereas acetylcholine-sensitivity was still evident several months after denervation.

**3** The catecholamine-induced contracture of the denervated muscle was inhibited specifically by $\beta$-receptor blocking drugs and was unaffected by $\alpha$-receptor blocking drugs and cholinoceptor antagonists.

**4** Catecholamine-induced contractures of denervated muscles, unlike contractures to acetylcholine, were dependent upon the presence of spontaneous fibrillation and the amplitude of spontaneous fibrillation was increased by catecholamines. Fibrillation was absent in the presence of tetrodotoxin (1 µM), 2,4-dinitrophenol (10 µM), potassium cyanide (10 µM), ouabain (100 µM), in lithium chloride Ringer solution and at low temperature. Under these conditions catecholamine-induced contractures, but not those to acetylcholine, were abolished.

**5** Labelled calcium was found progressively to enter denervated muscle fibres and this entry of calcium was increased by catecholamines. It is suggested that this calcium entry may represent either an increased calcium permeability of denervated muscle fibres which is increased further by catecholamines or the presence of a calcium current that occurs during the fibrillatory potentials of denervated muscle.

## Introduction

Chronically denervated mammalian skeletal muscle has been shown to produce contractures to applied catecholamines both *in vivo* (Bowman & Zaimis, 1961; Bowman & Raper, 1965; Turkanis, 1969) and *in vitro* (Montagu, 1955; Bhoola & Schachter, 1961; Paterson, 1963; Bhoola, Evans & Smith, 1972). The mechanism underlying this contractural response is poorly understood. Following denervation, mammalian skeletal muscle also produces a contracture in the presence of acetylcholine (Brown, 1937; Rosenbleuth & Luco, 1937). It has been demonstrated that this response is the result of development of cholinoceptors over the entire muscle fibre surface (Axelsson & Thesleff, 1959). The present experiments were designed to compare the pharmacology of the catecholamine-induced contracture of denervated muscle with the acetylcholine contracture, in order to see if there were any similarities in the mode of action of these drugs.

It was observed in the course of these experiments that the denervated muscle contracted in response to the catecholamines provided that the bathing fluid contained calcium. Similar findings were reported by Bhoola & Schachter (1961). Much evidence suggests that calcium ions play an important part in the linkage between excitation and contraction in muscles with normal resting potentials (see Sandow, 1965). Consequently, it was of special interest to study the calcium-dependence of these contractures and the effect of catecholamines on calcium fluxes.

The results obtained indicate that there may be a similarity between the response of denervated muscle to the catecholamines and the known effect of these drugs on the twitch tension of innervated muscle (Bowman & Zaimis, 1955, 1958; Bowman, Goldberg

---

[1] Present address: Department of Physiology, Queen Elizabeth College, Campden Hill Road, London W8 7AH.

& Raper, 1962) in that activation of fibres is a prerequisite for both processes. In denervated muscle the activation of fibres occurs spontaneously and is known as fibrillation (Tower, 1939). Some of these results have been presented in a preliminary form (Bhoola *et al.*, 1972).

## Methods

Diaphragm muscles from adult mice of either sex, body weight 25 to 35 g, were used. The diaphragms were denervated by intrathoracic section of the left phrenic nerve under chloroform anaesthesia at various intervals before the mice were killed. Isolated hemidiaphragms were suspended in a 10 ml organ bath which was filled with Ringer solution of the following composition (mM): NaCl 130, KCl 5, $NaH_2PO_4$ 1, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 12 and glucose 12. This solution was gassed with 95% $O_2$ and 5% $CO_2$ at all times and the temperature was maintained at $37 \pm 2°C$ unless stated otherwise. In some experiments the effects of lithium ions on the contractile response of denervated skeletal muscle to acetylcholine and isoprenaline and on the influx of calcium into denervated muscle was investigated. In these experiments lithium Ringer solution of the following composition was used (mM): LiCl 130, KCl 5, $NaH_2PO_4$ 1, $CaCl_2$ 2.5, $MgCl_2$ 1, $KHCO_3$ 12 and glucose 12.

Isometric tension developed by the muscles in response to drugs or electrical stimulation was measured with Sangamo displacement transducers (type D1/100) under an applied resting tension of 2 grams. The output from these transducers was led through a preamplifier with a pre-set gain of 1 to 10 into a Devices heated stylus pen recorder (type DC5H).

In order to stimulate muscles electrically, silver wire electrodes were placed at each end of the muscle and rectangular pulses of 1 ms duration and supramaximal intensity at a frequency of 0.05 Hz were applied.

To investigate the effect of isoprenaline and acetylcholine on calcium influx into denervated diaphragm muscles, paired segments of hemidiaphragms or groups of whole diaphragms were incubated for 3 min with $^{45}Ca$ Ringer solution and drug was then added to one of the segments or groups of muscles. After a given period of incubation, the muscles were rinsed briefly in tracer-free Ringer solution and the $^{45}Ca$ content was measured as described by Evans (1974).

The extent of the extracellular space in mouse diaphragm muscles was determined by the use of $^{35}SO_4$ as an extracellular marker (Walser, 1954; Robert, 1956). In these experiments, diaphragm muscles together with the rib cage were excised and incubated in Ringer solution to which $Na_2\,^{35}SO_4$ (0.1 µCi/ml Ringer) was added. After a given period of incubation, the diaphragm muscles were rinsed in $^{35}SO_4$-free Ringer solution, blotted, and dissected free of the rib cage and tendon. Each diaphragm was then separated into right and left hemidiaphragms and the wet weight of the hemidiaphragms was determined on a torsion balance. The weighed muscle samples were digested with 0.3 ml 0.3 M NaOH, then 2 ml of methanol and 10 ml of phosphor (2, methoxyethanol, 3 litres, toluene 7 litres, 2,5-diphenyloxazole 40 g, 1:4-di-2 (5-phenyloxazolyl)-benzene 1 g) was added to the samples before measurement of $^{35}S$ by liquid scintillation counting. The $^{35}SO_4$ space was calculated as

$$\frac{\text{ct/min per g wet weight of muscle}}{\text{ct/min per ml of Ringer}}$$

## Results

### *Characteristics of the contractural response*

Responses of denervated mouse diaphragm muscle to acetylcholine and (±)-isoprenaline are shown in Figure 1a, and the time course of contracture in the continued presence of both drugs is illustrated in Figure 1b. It can be seen that the response to isoprenaline is slower in onset and has longer duration than that to acetylcholine. The thickness of the base-line in Figure 1a and in subsequent records was produced by the spontaneous fibrillatory activity of the muscles. The catecholamine contractural response was accompanied by an increase in fibrillatory activity which can be seen as the increased amplitude of the base-line that occurs at the peaks of the isoprenaline responses in Figure 1a. It was found that the increase in tension and fibrillation produced by the application of isoprenaline to denervated diaphragm muscle was maintained for up to 45 min following washout of the drug. Tachyphylaxis to successive doses of isoprenaline given at intervals of less than 30 min was observed, and there appeared to be a deterioration of the catecholamine response compared to the acetylcholine response in tissues that had been mounted in Ringer solution for longer than six hours.

The response of denervated mouse diaphragm to the catecholamines was not always a simple increase in tension. Occasionally the response was biphasic and the contraction was preceded by a small and short lasting reduction in tone. Similar findings were reported by Bowman & Raper (1965) for denervated soleus and tibialis anterior muscles of the cat *in vivo*.

The maximum tension developed by denervated mouse hemidiaphragm muscles to acetylcholine was 1.71 g ± 0.16 s.e. mean ($n = 5$). Isometric tensions produced by isoprenaline were smaller and were usually about 20% of those produced by acetylcholine.

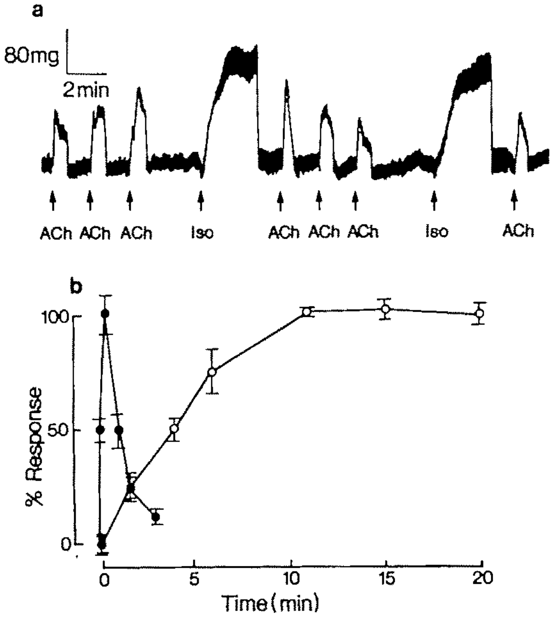


**Figure 1** (a) Contractural responses of denervated diaphragm muscle of mouse evoked by acetylcholine, 5 μM (ACh) and isoprenaline, 3 μM (Iso). The recording was stopped 1 min after addition of acetylcholine and 2 min after addition of isoprenaline to the bathing fluid and fresh Ringer solution perfused until the tension had returned to the base-line. (b) Time course of the change in isometric tension of denervated mouse diaphragm muscle in the presence of acetylcholine and isoprenaline. The percentage of the maximum tension developed is plotted against time after application of drug. (●) Acetylcholine, 4 μM; (O) isoprenaline, 2.5 μM. Each point is the mean response of five muscles. Vertical lines show s.e. mean.

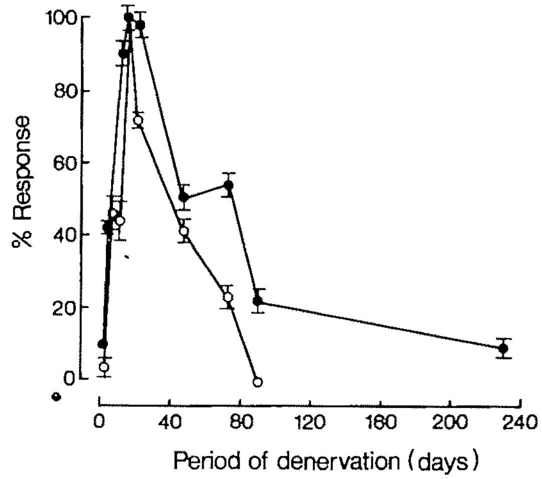


**Figure 2** Time course of the development of sensitivity to acetylcholine and isoprenaline of denervated diaphragm muscle of mouse. Maximum contractural responses to 2 mM acetylcholine (●) and 2.5 μM isoprenaline (O) expressed as a percentage of the mean response at 15 days are plotted against time after denervation. Each point is the mean response of five muscles. Vertical lines show s.e. mean.

### *Development of the contractural response*

In order to see if catecholamine-sensitivity of denervated diaphragm muscle developed in a similar way to acetylcholine-sensitivity, maximum contractural responses of denervated mouse diaphragm muscle to isoprenaline and acetylcholine in Ringer solution at 37°C were measured at various times after denervation. Isoprenaline was chosen as the most suitable catecholamine stimulus because the tissue showed highest specificity towards this catecholamine (see Figure 3). Figure 2 shows the time course of the development of the response to both drugs. Two days after denervation both acetylcholine and isoprenaline produced detectable contractural responses in the denervated muscle. The maximum effect of both drugs appeared after 14 days denervation. However, isoprenaline-induced contractures were absent in muscles that had been denervated for longer than 90 days, whereas acetylcholine contractures were still present in muscles 230 days after denervation.

The fact that isoprenaline contractures were absent in muscles which still responded to acetylcholine, suggested that the development of sensitivity to acetylcholine and to isoprenaline in denervated diaphragm muscles involved separate processes. Further indication of the difference between the development of acetylcholine and isoprenaline-sensitivity in skeletal muscle was obtained from studies on foetal and neonatal tissues. In these experiments it was found that whereas acetylcholine produced contractures in diaphragms taken from eight 22-day old foetal and six 2 to 3 day old neonatal rats, no contractures to isoprenaline were observed in these tissues.

### *Specificity of the contractural responses to acetylcholine and the catecholamines*

To show how specific the contractural responses of denervated muscles to acetylcholine and isoprenaline were, the effects of several other potential agonists were tested. It was found that denervated mouse diaphragm muscle failed to contract in the presence of histamine (10 μM), 5-hydroxytryptamine (10 μM), bradykinin (0.01 μM), vasopressin (0.2 u/ml), $\gamma$-aminobutyric acid (2 mM) and L-glutamate (2 mM). However, the cholinomimetic drugs carbachol,

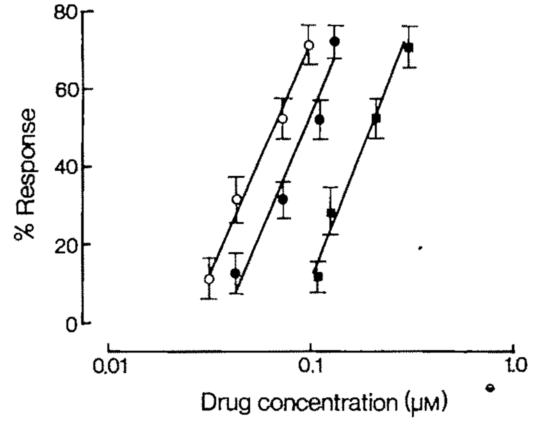


**Figure 3** Dose-response curves of denervated diaphragm muscle of mouse to (±)-isoprenaline (O), (−)-adrenaline (●) and (−)-noradrenaline (■). Each point is the mean result from at least four muscles. Vertical lines show s.e. mean.

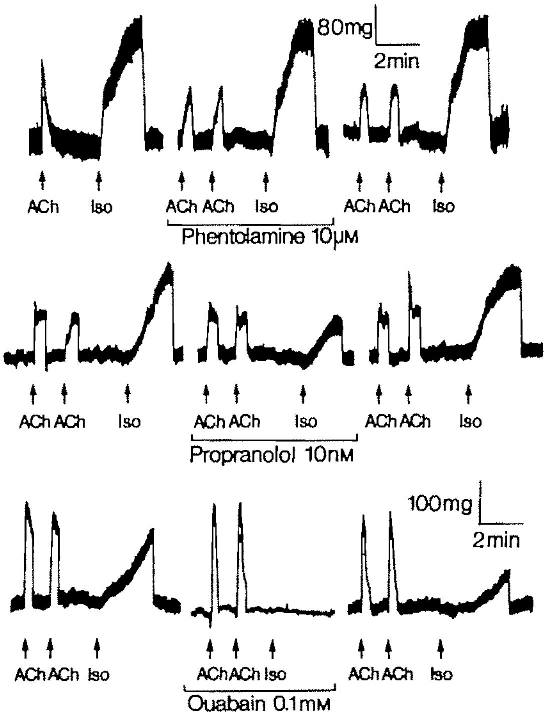


**Figure 4** The effect of propranolol, phentolamine and ouabain on contractural responses of denervated diaphragm muscle of mouse evoked by acetylcholine, 5 μM (ACh), and isoprenaline, 2.5 μM (Iso). The muscles were preincubated with propranolol or phentolamine for 2 min or with ouabain for 5 min and were then treated with acetylcholine or isoprenaline in the presence of the antagonist. Responses recorded as for Figure 1(a).

butyrylcholine and nicotine, and the three catecholamines, (−)-noradrenaline, (−)-adrenaline and (±)-isoprenaline all produced dose-dependent increases in tension in the denervated muscle. Figure 3 compares dose-response relations of denervated diaphragm muscles to the three catecholamines. The tension produced by each drug is plotted as a percentage of the maximum response. On a molar basis isoprenaline was one and a half times as potent as adrenaline which was in turn twice as potent as noradrenaline.

The increase in tension produced by adrenaline and isoprenaline was unaltered by (±)-tubocurarine (7 μM) and atropine (20 μM) in concentrations that blocked the acetylcholine response. The contractural responses of denervated muscles to isoprenaline and adrenaline were unaltered also by the α-adrenoceptor blocking agents phentolamine (Figure 4a) and thymoxamine (1 μM to 10 μM). However, catecholamine contractures of denervated diaphragm muscles were blocked by the β-adrenoceptor antagonists propranolol (Figure 4b), sotalol and methoxamine (0.01 μM to 10 μM) added either before or in the presence of the agonist. These results suggest that denervated mouse diaphragm muscle possesses β-adrenoceptors that are separate from cholinoreceptors.

The relationship of β-adrenoceptive effects to cyclicadenosine 3′,5′-monophosphate (cyclic AMP) levels is well documented (Robison, Butcher & Sutherland, 1971) and Bowman & Nott (1969) have suggested that cyclic AMP is involved in the adrenoceptive processes of skeletal muscle fibres. However, in the present experiments, neither cyclic AMP nor the dibutyryl ester of cyclic AMP (10 μM to 1 mM) produced any effect on the tension of denervated muscles or modified the contractures produced by either acetylcholine or catecholamines.

Catecholamines are known to stimulate glycogenolysis in skeletal muscle as in most tissues via a cyclic AMP-mediated process (Mayer & Stull, 1971). However, it is unlikely that this process is immediately essential for the catecholamine-induced contracture of denervated muscles because it was found that substitution of sucrose for glucose in the Ringer solution abolished isoprenaline-induced contractures within 15 minutes. On restoration of glucose, isoprenaline-induced contractures reappeared. Acetylcholine-induced contractures were not abolished by this treatment. If catecholamine-induced contractures were a direct result of increased glycogenolysis such immediate dependence on the external glucose would not be expected.

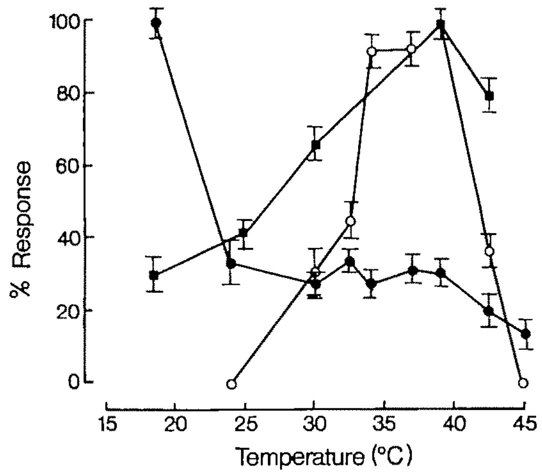


**Figure 5** The effect of temperature on drug-induced contractural responses of denervated diaphragm muscle of mouse and on the fibrillatory activity of the muscle. (O) Tension developed in the presence of isoprenaline, 2.5 μM; (●) tension developed in the presence of acetylcholine, 3 μM; (■) amplitude of the fibrillatory activity of the muscle. Each point is the mean result from at least four muscles. Vertical lines show s.e. mean.

### *Effect of temperature, metabolic inhibitors, cardiac glycosides and lithium*

Figure 5 shows the effect of temperature on the response of denervated muscle to acetylcholine and isoprenaline. It can be seen that the response to acetylcholine was greater at 18°C than at 37°C. Similar findings have been reported by Letley (1960) and Freeman & Turner (1969). On the other hand, the isoprenaline response showed a definite peak at 37°C as did the amplitude of the fibrillatory activity of the muscle. To investigate this further the response of denervated muscles to acetylcholine and isoprenaline was measured after treatment with metabolic inhibitors potassium cyanide (10 μM) and 2,4-dinitrophenol (10 μM). It was found that both metabolic inhibitors increased the resting tension and abolished the fibrillatory activity of the denervated muscle. In the presence of 2,4-dinitrophenol and potassium cyanide, isoprenaline-induced contractures of the denervated muscle were absent whereas acetylcholine contractures were unaffected or only partially inhibited. After removal of potassium cyanide from the muscle bath responses to isoprenaline reappeared after 15 minutes. However, the effects of 2,4-dinitrophenol were found to be irreversible.

The effects of glucose, 2,4-dinitrophenol, potassium cyanide and temperature suggested that the response of denervated muscle to isoprenaline was immediately dependent on oxidative metabolism. To test whether this dependence on oxidative metabolism was related to the action of the sodium pump of the muscle membrane, the effects of lithium and the cardiac glycosides ouabain and strophanthin K were investigated. Addition of ouabain (100 μM) or strophanthin K (100 μM) to the organ bath resulted in a rapid decrease (within 7 min) in the resting tension and fibrillatory activity of the denervated muscle. The introduction of lithium Ringer solution produced a gradual increase in the resting tension which reached its peak within 20–30 minutes. This increase in tension was accompanied by a decrease in the fibrillatory activity of the muscle. Preincubation of the denervated muscle with ouabain (Figure 4c) or strophanthin K for 15 min, or in lithium Ringer solution for 2 h abolished responses to isoprenaline but had little effect on acetylcholine contractures.

### *Effect of tetrodotoxin*

Katz & Miledi (1967) found that depolarization of the extra-junctional membrane of denervated muscle produced by acetylcholine was insensitive to tetrodotoxin. On the other hand, the generation of action potentials in innervated muscle is blocked by tetrodotoxin (Narahashi, Deguchi, Urakawa & Ohkulo, 1960), which appears to inhibit specifically the inward sodium current (Kao, 1966). To see if the action of tetrodotoxin would provide a clue to the mechanism of the catecholamine-evoked contracture of denervated muscle, the effects of this drug were compared on both denervated and innervated diaphragm preparations. It was found that tetrodotoxin at a concentration of 3 μM completely blocked the innervated preparation to direct and indirect stimulation within 15 minutes. Similar doses of tetrodotoxin caused a decrease in resting tension of the denervated diaphragm and abolished both fibrillatory activity and isoprenaline-induced contractures. However, even after exposure for 60 min, tetrodotoxin (3 μM) was without effect on acetylcholine contractures.

The finding that the response to acetylcholine of denervated diaphragm muscle was totally unaffected by concentrations of tetrodotoxin that rapidly blocked the innervated preparation, provides confirmation for the view that the acetylcholine contracture of denervated skeletal muscle is the result of non-propagated depolarization of the muscle membrane (see Thesleff, 1960). However, the observation that isoprenaline contractures of the denervated muscle were absent in the presence of tetrodotoxin indicates that generation of the muscle fibre action potential is a prerequisite for the catecholamine response.

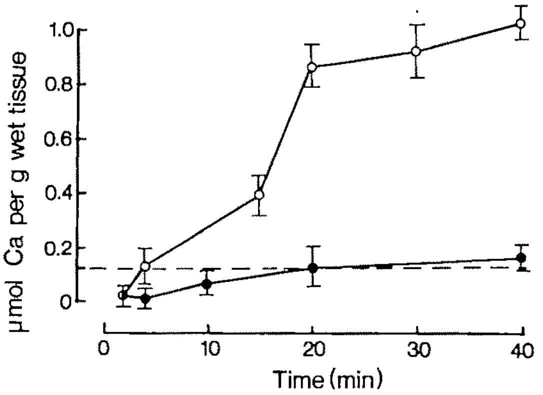


**Figure 6** The time course of accumulation of calcium by denervated and innervated diaphragm muscles of mouse. Diaphragms were incubated in $^{45}$Ca Ringer solution at 37°C for the intervals indicated and were then prepared for liquid scintillation counting as described in the Methods section. (O) Calcium accumulated in denervated muscles; (●) calcium accumulated in innervated muscles. Each point is the mean result from six muscles. Vertical lines show s.e. mean. The dashed line indicates the level of $^{45}$Ca contained by the extracellular space.

### *Entry of calcium*

Bhoola & Schachter (1961) reported that catecholamine-evoked contractures of denervated rat diaphragm muscle were dependent upon the presence of calcium in the Ringer solution. In the present experiments the dependence of isoprenaline contractures, acetylcholine contractures and contractions caused by direct electrical stimulation of muscles, on the calcium concentration of the Ringer solution was examined. After 45 min in calcium-free Ringer solution, the drug-induced contractures were absent but contractions caused by electrical stimulation were still evident after 2 hours. Thus the drug-induced contractures were specifically dependent on the presence of external calcium ions.

The influence of external calcium on the drug-induced contractures was investigated further by incubating muscles in Ringer containing $^{45}$CaCl$_2$ as a tracer. First, the resting entry of $^{45}$Ca into denervated and innervated sides of diaphragm muscles was compared. To determine whether $^{45}$Ca entered muscle fibres, the entry of $^{45}$Ca was compared with the entry of $^{35}$SO$_4$. The entry of $^{35}$SO$_4$ into muscles gave a value for the extracellular space of the innervated sides of muscles of 0.25 ± 0.02, and 0.28 ± 0.02 s.e. mean ($n = 10$) for the denervated sides. These values were not significantly altered by drug treatment. The entry of $^{45}$Ca into the innervated sides of muscles reached a plateau between 10 and 20 min after placing muscles in $^{45}$Ca Ringer solution and this plateau level corresponded with the $^{35}$SO$_4$ space shown by the dashed line in Figure 6. However, on the denervated side there was a progressive entry of $^{45}$Ca into muscle fibres (upper curve Figure 6).

Table 1 shows the effect of 4 and 20 min incubation with 2.5 μM isoprenaline on the entry of $^{45}$Ca into paired segments of denervated hemidiaphragms. Treatment with isoprenaline for 4 min caused a threefold increase in the entry of $^{45}$Ca into muscle fibres ($P < 0.001$). A similar effect was observed with adrenaline (2.7 μM). When denervated muscles were incubated in $^{45}$Ca Ringer solution for 2 h and then placed in tracer-free Ringer solution, they lost 30% of the tracer in 4 minutes. Addition of isoprenaline (2.5 μM) to the washout solution had no significant effect on this loss of $^{45}$Ca. Therefore, a net increase in the influx of calcium into the muscle fibres was caused by isoprenaline. The initial rate of influx of calcium caused by isoprenaline could not have been sustained because after 20 min the amount of $^{45}$Ca in drug treated tissues was not significantly different from controls (Table 1).

**Table 1** Effect of isoprenaline (2.5 μM) on accumulation of $^{45}$Ca in denervated mouse diaphragm muscles

| *Incubation time (min)* | *μmol Ca/g wet tissue* Control muscles | Treated muscles |
|---|---|---|
| 4 | 0.15 ± 0.01 | 0.53 ± 0.06 |
| 20 | 0.87 ± 0.05 | 0.94 ± 0.06 |

Values are mean of 18 muscles ± s.e. mean.

In innervated muscles no catecholamine-induced influx of labelled calcium was observed either at the junctional region or in the non-innervated segments. Furthermore, the isoprenaline-induced influx of $^{45}$Ca did not occur in muscles that had been denervated for 100 days, or in denervated muscles that were equilibrated with lithium Ringer solution, in the presence of ouabain (100 μM) or propranolol (1 μM), or at 18°C, all of these being experimental conditions in which the contractural response is absent.

## Discussion

In the present study it was found that the increase in tension produced by the catecholamines in denervated mouse diaphragm muscles was accompanied by an increase in the fibrillatory activity of the muscles. Both the contractural response and increase in fibrillatory activity were related in terms of the relative potency of the catecholamines and sensitivity to antagonists. A catecholamine-induced increase in electrical activity associated with fibrillation of denervated muscle has been reported elsewhere (Bowman & Raper, 1965;

Smith & Thesleff, 1975). Bowman & Raper (1965) suggested that there was a causal relationship between the catecholamine-evoked increase in the frequency of fibrillatory potentials and contracture. The present evidence supports this view because the absence of isoprenaline-evoked contractures of denervated muscle in the presence of tetrodotoxin indicates very clearly the role of propagated action potentials in the increase in tension. The evidence from calcium flux measurements is supportive also in this respect because these show that the catecholamine-induced contracture of denervated diaphragm muscle is accompanied by an influx of calcium, which is known to enter the muscle fibres during activity (Bianchi & Shanes, 1959; Evans, 1974).

The 37°C optimum for the catecholamine response and the effects of 2,4-dinitrophenol, potassium cyanide, ouabain, strophanthin K and lithium could suggest a role for the sodium pump in the catecholamine-induced contracture of denervated muscle. This may be so, but Bowman & Raper (1964) showed that the frequency of fibrillatory potentials in denervated cat muscles was reduced by lowering the temperature and in the present study it was found that 2,4-dinitrophenol, potassium cyanide, ouabain, strophanthin K and lithium all blocked the catecholamine response and abolished fibrillatory activity. Therefore, it is likely that the important effect of these substances, in so far as the response to catecholamines is concerned, is the abolition of fibrillation.

Fibrillation of denervated muscles results from asynchronous contraction of muscle fibres and we have observed that only a fraction of denervated mouse diaphragm muscle fibres are contracting at any one time. Purves & Sakmann (1974) have estimated this fraction of active fibres to be about one-third for denervated rat diaphragm muscles. The results of Evans (1974) show that approximately 1 nmol Ca/mg dry muscle entered innervated mouse diaphragm muscles during a 15 min period of supramaximal tetanic stimulation. Thus if calcium entry per impulse is similar in denervated and innervated muscles then a value somewhat less than this would be expected for calcium entry into unstimulated denervated muscles. However, the present results (Figure 6) yield a value of 1.0 nmol Ca/mg dry unstimulated muscles per 15 min, and there was an initial fourfold increase in this level of calcium entry after treatment with isoprenaline. Therefore, it appears either that the resting permeability to calcium is higher in denervated fibres than in innervated fibres, and that this is increased still further by isoprenaline, or that denervated fibres have a much higher level of calcium entry per propagated impulse than occurs in innervated fibres.

At this stage it is possible to indicate some relationship between the increased tension of denervated muscles and the increased twitch tension of innervated muscles produced by the catecholamines (Bowman & Zaimis, 1955, 1958; Bowman *et al.*, 1962). In both cases muscle fibres have to be activated before any increase in tension can be shown. In the case of denervated muscle the activation is spontaneous but may be abolished by tetrodotoxin, which indicates the role of propagated action potentials in the increase in tension. In the case of innervated muscle there is no spontaneous activity and increased tension in the presence of catecholamines is only seen when muscles are stimulated electrically.

The present results indicate clearly that in denervated mouse diaphragm muscles the responses to catecholamines were not caused by reaction with cholinoceptors but with specific $\beta$-adrenoceptors, because they were produced most effectively by isoprenaline and were selectively blocked by $\beta$-receptor blocking drugs. This is comparable to the effect of catecholamines on denervated muscles of the cat, *in vivo* (Bowman & Raper, 1965). However, Paterson (1963) reported that in isolated denervated diaphragm of the rat the contracture evoked by adrenaline was blocked most effectively by $\alpha$-receptor blocking drugs, while Yamada & Harigaya (1974) reported a biphasic contractural response to adrenaline elicited through both $\alpha$- and $\beta$-receptors in denervated extensor digitorum longus and soleus muscles of the rat. We cannot explain the discrepancy between these results.

Comparison of the time course of development of acetylcholine- and isoprenaline-induced contractures in denervated mouse diaphragm muscles showed that after long periods of denervation, responses could be obtained to acetylcholine but not to isoprenaline. This again suggests that the sensitivity of denervated muscles to acetylcholine and the catecholamines are two separate receptive processes. The observation that foetal and neonatal tissues produced contractures in the presence of acetylcholine but not isoprenaline supports this view. In tissues denervated for long periods, in which the contractural response to isoprenaline was absent, and in the foetal and neonatal tissues studied, fibrillatory activity was undiscernible. This observation provides a further confirmation for the view that fibrillation is an integral part of the process involved in the catecholamine contracture of denervated skeletal muscle. However, the problem of the relationship between the $\beta$-adrenoceptor and the process which initiates spontaneous action potentials in denervated muscle fibres remains unsolved although the high resting influx of calcium in denervated muscles suggests the possibility that spontaneous inward calcium currents may play a trigger role.

This work was supported by the National Fund for Research into Crippling Diseases.

**References**

AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol., Lond.*, **147**, 178–193.

BHOOLA, K.D., EVANS, R.H. & SMITH, J.W. (1972). Catecholamine induced contractures in denervated muscle. *Br. J. Pharmac.*, **46**, 531–532P.

BHOOLA, K.D. & SCHACHTER, M. (1961). Contracture of the denervated rat diaphragm by adrenaline. *J. Physiol., Lond.*, **157**, 20–21P.

BIANCHI, C.P. & SHANES, A.M. (1959). Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. *J. gen. Physiol.*, **42**, 803–815.

BOWMAN, W.C., GOLDBERG, A.A.J. & RAPER, C. (1962). A comparison between the effects of a tetanus and the effects of sympathomimetic amines on fast and slow contracting mammalian muscles. *Br. J. Pharmac., Chemother.* **19**, 464–484.

BOWMAN, W.C. & NOTT, M.W. (1969). Sympathomimetic amines on skeletal muscle. *Pharmac. Rev.*, **21**, 27–72.

BOWMAN, W.C. & RAPER, C. (1964). Spontaneous fibrillary activity of denervated muscle. *Nature, Lond.*, **193**, 41–43.

BOWMAN, W.C. & RAPER, C. (1965). The effects of sympathomimetic amines on chronically denervated skeletal muscle. *Br. J. Pharmac. Chemother.*, **24**, 98–109.

BOWMAN, W.C. & ZAIMIS, E. (1955). A comparison between the responses of the tibialis anterior and the soleus muscles in the cat to adrenaline, noradrenaline and isoprenaline. *J. Physiol., Lond.*, **128**, 14–15P.

BOWMAN, W.C. & ZAIMIS, E. (1958). The effects of adrenaline, noradrenaline and isoprenaline on skeletal muscle contractions in the cat. *J. Physiol., Lond.*, **144**, 92–107.

BOWMAN, W.C. & ZAIMIS, E. (1961). The action of adrenaline, noradrenaline and isoprenaline on the denervated mammalian muscle. *J. Physiol., Lond.*, **158**, 24–25P.

BROWN, G.L. (1937). The action of acetylcholine on denervated mammalian and frog muscle. *J. Physiol., Lond.*, **89**, 438–461.

EVANS, R.H. (1974). The entry of labelled calcium into the innervated region of mouse diaphragm muscle. *J. Physiol. Lond.*, **240**, 517–533.

FREEMAN, S.E. & TURNER, R.J. (1969). Ionic interactions in acetylcholine contraction of the denervated rat diaphragm. *Br. J. Pharmac.*, **36**, 510–522.

KAO, C.Y. (1966). Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmac. Rev.*, **18**, 997–1050.

KATZ, B. & MILEDI, R. (1967). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol., Lond.*, **192**, 407–436.

LETLEY, E. (1960). The effect of temperature on the direct muscle twitch response and the action of drugs on the isolated denervated rat diaphragm. *Br. J. Pharmac. Chemother.*, **15**, 345–350.

MAYER, S.E. & STULL, J.T. (1971). Cyclic AMP in skeletal muscle. *Ann. N.Y. Acad. Sci.*, **185**, 433–448.

MONTAGU, K.A. (1955). On the mechanisms of action of adrenaline in skeletal nerve-muscle. *J. Physiol., Lond.*, **128**, 619–628.

NARAHASHI, T., DEGUCHI, T., URAKAWA, N. & OHKULO, Y. (1960). Stabilisation and rectification of muscle fibre membrane by tetrodotoxin. *Am. J. Physiol.*, **198**, 934–938.

PATERSON, G. (1963). The dual action of adrenaline on denervated skeletal muscle. *Biochem. Pharmac.*, **12**, 85.

PURVES, D. & SAKMANN, B. (1974). The effect of contractile activity on fibrillation and extrajunctional acetylcholine sensitivity in rat muscle maintained in organ culture. *J. Physiol., Lond.*, **237**, 157–182.

ROBERT, J. (1956). Radiosulphate space as an index of extracellular water. *J. clin. Invest.*, **35**, 1119–1130.

ROBINSON, G.A., BUTCHER, R.W. & SUTHERLAND, E.W. (1971). *Cyclic AMP*. New York: Academic Press.

ROSENBLEUTH, A. & LUCO, J.U. (1937). A study of denervated mammalian skeletal muscle. *Am. J. Physiol.*, **120**, 781–797.

SANDOW, A. (1965). Excitation-contraction coupling in skeletal muscle. *Pharmac. Rev.*, **17**, 265–320.

SMITH, J.W. & THESLEFF, S. (1975). Spontaneous activity in denervated mouse diaphragm muscle. *J. Physiol., Lond.*, **257**, 171–187.

THESLEFF, S. (1960). Effects of motor innervation on the chemical sensitivity of skeletal muscle. *Physiol. Rev.*, **40**, 734–752.

TOWER, S.S. (1939). The reaction of muscle to denervation. *Physiol. Rev.*, **19**, 1–48.

TURKANIS, S.A. (1969). Some properties of the denervated anterior gracilis muscle of the rat. *Br. J. Pharmac.*, **37**, 414–424.

WALSER, M. (1954). Radiosulphate space of muscle. *Am. J. Physiol.*, **176**, 322–324.

YAMADA, K. & HARIGAYA, S. (1974). Contractile responses to sympathomimetic amines in isolated rat muscles after chronic denervation. *Japan. J. Pharmac.*, **24**, 487–490.

*(Received February 9, 1976.*
*Revised April 5, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 117–119

# EFFECTS OF L-TRYPTOPHAN AND L-METHIONINE ON ACTIVITY IN THE RAT

M. TAYLOR
Department of Psychology, Concordia University, Montreal, Quebec, Canada

1 The effects of an intraperitoneal dose of 20 mg/kg L-tryptophan and doses of 100 and 150 mg/kg of L-methionine were investigated on activity in the rat.

2 Activity was measured by a time sampling behaviour categorization procedure, and began 15 min after compound administration, lasting for a total of one hour.

3 Total active behaviour in the first half hour was reduced after 20 mg/kg tryptophan, 100 or 150 mg/kg methionine. Only 20 mg/kg tryptophan led to a significant reduction in active behaviour during the second half hour.

4 Administration of 100 mg/kg methionine plus 20 mg/kg tryptophan, and 150 mg/kg methionine plus 20 mg/kg tryptophan had no discernible effect on activity.

5 The results draw attention to the role of another amino acid in the modification of the behavioural effects of tryptophan.

## Introduction

There have been a number of recent investigations of the effects of various amino acids on behaviour. Many of these investigations have concentrated on the essential amino acids tryptophan (e.g. Modigh, 1973; Hingtgen & Aprison, 1975) and methionine (e.g. Beaton, 1975), and have often involved administration of relatively large quantities, in relation to normal dietary intake.

Both amino acids are of considerable potential behavioural interest. Tryptophan is metabolized along several pathways, which include the formation of tryptamine and 5-hydroxytryptamine (5-HT). 5-HT is implicated in a wide range of behavioural processes such as hunger regulation, pain sensitivity, etc. Changes in blood tryptophan have been shown to be associated with changes in brain 5-HT (Wurtman & Fernstrom, 1975), but whilst such central changes are suggestive, they do not necessarily imply appreciable behavioural effects (Grahame-Smith, 1973). Methionine has been suggested to have a possible role in the aetiology of some forms of schizophrenia (Osmund & Smithies, 1952) where its properties as a methyl donor have been emphasized.

In general, both amino acids have been reported to reduce motor activity. The dose of tryptophan found to achieve this effect has varied between 0.7 mg/kg (Brown, 1960) and 800 mg/kg (Modigh, 1973) after intraperitoneal injection. However, as in numerous other investigations of effects on activity, this variation may in part be a function of the methods used for recording activity.

Taylor, Goudie & Williams (1973), Goudie & Taylor (1974) and Taylor, Goudie, Mortimore & Wheeler (1974) have reported a technique for time sampling activity in rats. A modification of this technique is described here and was used to investigate the effects on activity of a single dose of 20 mg/kg L-tryptophan, and of 100 and 150 mg/kg L-methionine, both singly and in combination with tryptophan.

## Methods

Male Wistar albino rats were used, housed individually, and weighing approximately 300 grams. The subjects were housed under normal lighting conditions with exposure to food and water *ad libitum*. An experimental group was made up of 7 randomly chosen rats. Six experimental groups were used: (1) control—2 injections of vehicle (distilled water); (2) 20 mg/kg tryptophan—tryptophan plus one injection of vehicle; (3) 100 mg/kg methionine and (4) 150 mg/kg methionine—appropriate dose of methionine plus an injection of vehicle; (5) 100 mg/kg methionine plus 20 mg/kg tryptophan and (6) 150 mg/kg methionine plus 20 mg/kg tryptophan—appropriate injections of both compounds. All compounds were administered in a

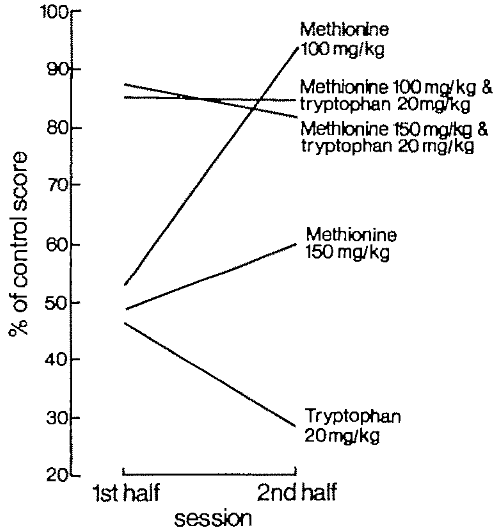


**Figure 1** Active behaviour of rats after injections of tryptophan, methionine or both amino acids during the first and second half sessions expressed as a percentage of control values.

volume of 2 ml/kg by intraperitoneal injection, and were made up in distilled water. The pH of all solutions administered was approximately 7.

Observations were conducted between 12 h 00 min and 18 h 00 min on successive days. Immediately after injection, rats were individually placed in an evenly illuminated open field (45 cm x 45 cm) where their behaviour was observed and recorded for 1 min intervals every 5 min, beginning 15 min after injection. A time sampling 'all-or-nothing' system of recording activity was used, based on Taylor *et al.* (1974) and Goudie & Taylor (1974). The occurrence of any of the following behaviours was recorded during the 1 min observation period: rearing, walking, sniffing, grooming, immobility (see Taylor *et al.*, 1973, for details of behaviours). Frequency of occurrence of the behaviours within the 1 min interval was not recorded. The summation of the categories rearing and walking were defined as 'active' behaviours. Observations were conducted until 75 min post injection.

## Results

Figure 1 shows the active behaviour observed during the first half (30 min) and second half (30 min) sessions, expressed as a percentage of control values. Both doses of methionine and the dose of tryptophan used, when compared to controls, led to a significant reduction in total active behaviour for the first half of the sessions (Mann-Whitney U test: $P < 0.05$). Only tryptophan remained significantly different from control during the second half session, although a near significant reduction in the 150 mg/kg methionine group ($P = 0.064$) is apparent. Tryptophan administered with either dose of methionine produced no significant effects on behaviour during either the first or second half session, with no evidence of a dose-response relationship.

No significant differences were found in the incidence of immobile grooming and sniffing behaviours between the various groups. Informal observations of animals revealed no systematic effects, other than a general reduction in activity corresponding to the above differences.

## Discussion

The results presented here suggest that a dose of 20 mg/kg L-tryptophan is sufficient to have an effect upon behaviour in terms of a reduction in active categories. Because a smaller dose than that reported here has been shown to increase brain 5-HT (Wurtman & Fernstrom, 1975), it is tempting to suggest that the behavioural effect noted is related to such an increase. 5-HT and its immediate precursor 5-hydroxytryptophan, have been reported to produce behavioural depression in rats (Aprison & Hingtgen, 1966); on the other hand, there is evidence that the effects on motor activity of 5-HT and 5-hydroxytryptophan are at least in part the result of peripheral rather than central factors (e.g. Jacobs & Eubanks, 1974).

Wayner, Ono, Deyoung & Barone (1975) have demonstrated that changes in extracellular reaction potentials of cells in various brain areas can occur when tryptophan and methionine, among other essential amino acids, are administered microelectrophoretically. This introduces the possibility of some relatively direct central action of the amino acids, as well as potential central actions of metabolites e.g. 5-HT.

Methionine at both doses used here produced a reduction in active behaviour during the first half session, of comparable magnitude to that produced by tryptophan; no significant effects were apparent in the second half session, although there is a non-significant suggestion of a dose-related effect, in terms of duration of action in the 150 mg/kg group. The dose levels used here are somewhat lower than those reported by other authors (Bovet, Leathwood, Mauron, Olivero & Satta, 1971; Beaton, 1975). Whilst the specific central action of methionine remains obscure, there is evidence that L-DOPA acts as a competitive inhibitor of methionine uptake by nerve endings in homogenates of rat brains (Baldessarini & Karobath, 1972). Furthermore, methionine appears to reverse the clinical effectiveness of L-DOPA in Parkinsonism (Pearce & Waterbury, 1974). These reports suggest some interaction between methionine and L-DOPA, which may perhaps mediate some of the behavioural effects described here.

Administration of both compounds together failed to produce any behavioural effects. Neither the dose of methionine nor the length of time after administration appear to be relevant factors, which is surprising in view of their effects when the compounds are administered singly.

Wurtman & Fernstrom (1975) have demonstrated that brain 5-HT and tryptophan levels reflect not only plasma concentrations of tryptophan, but also plasma concentrations of other amino acids. An analagous process might be suggested here, as some form of mutual interference after the administration of tryptophan together with methionine. Whether such interference involves a common transport system, or operates at some other level is not clear. However, these results emphasize the role of another amino acid in the modification of the behavioural effects of tryptophan.

This work was supported by a grant from the Schizophrenia Association of Great Britain. The author is on leave of absence from the Department of Psychology, University College of North Wales, Bangor, U.K.

**References**

APRISON, M.H. & HINGTGEN, J.N. (1966). Neurochemical correlates of behaviour V. Differential effects of drugs on approach and avoidance behavior in rats with related changes in brain serotonin and norepinephrine. *Recent Adv. Biol. Psychiat.*, **8**, 87–100.

BALDESSARINI, R.J. & KAROBATH, M. (1972). Effects of L-DOPA and L-3-0-methyl-dopa on uptake of (3H) L-methionine by synaptosomes. *Neuropharmac.*, **11**, 715–720.

BEATON, J.M. (1975). Methylation and schizophrenia. *Alabama J. Med. Sc.*, **12**, 193–202.

BOVET, D., LEATHWOOD, P., MAURON, J., OLIVERIO, A. & SATTA, M. (1971). The effects of different amino acid diets on a fast-induced performance decrement in mice. *Psychopharmacologia, (Berl)*, **22**, 91–99.

BROWN, B.B. (1960). CNS drug actions and interactions in mice. *Arch int. Pharmacodyn.*, **128**, 391–414.

GOUDIE, A.J. & TAYLOR, M. (1974). Time sampling of rat exploratory behavior: a reliable screening test for the CNS effects of anorectic drugs. *Psychopharmacologia (Berl)*, **31**, 1–12.

GRAHAME-SMITH, D.G. (1973). Does the total turnover of brain 5-HT reflect the functional activity of 5-HT in brain? In *Serotonin and Behavior*, 5–7, ed. Barchas, J. & Usdin, E. New York and London: Academic Press.

HINGTGEN, J.N. & APRISON, M.H. (1975). Behavioural depression following L-tryptophan administration. *Life Sciences*, **16**, 1471–1476.

JACOBS, B.L. & EUBANKS, E.E. (1974). A comparison of the locomotor effects of 5-hydroxytryptamine and 5-hydroxytryptophan administered via two systemic routes. *Pharmacol. Biochem. and Behav.*, **2**, 137–139.

MODIGH, K. (1973). Effects of L-tryptophan on motor activity in mice. *Psychopharmacologia (Berl)*, **30**, 123–134.

OSMUND, H. & SMYTHIES, J.R. (1952). Schizophrenia: a new approach. *J. Ment. Sci.*, **98**, 309–315.

PEARCE, L.A. & WATERBURY, L.D. (1974). L-Methionine: a possible levodopa antagonist. *Neurology*, **24**, 640–641.

TAYLOR, M., GOUDIE, A.J. & WILLIAMS, A. (1973). Effects of chronic fenfluramine administration on behavior and body weight. *Psychopharmacologia, (Berl)*, **31**, 63–76.

TAYLOR, M., GOUDIE, A.J., MORTIMORE, S. & WHEELER, T.J. (1974). Comparisons between behaviors elicited by high doses of amphetamine and fenfluramine: implications for the concept of stereotypy. *Psychopharmacologia (Berl)*, **40**, 249–257.

WAYNER, M.J., ONO, T., DEYOUNG, A. & BARONE, F.C. (1975). Effects of amino acids on central neurons. *Pharmacol. Biochem. and Behav.*, **3**, Suppl. 1, 85–90.

WURTMAN, R.J. & FERNSTROM, J.D. (1975). Control of brain monoamine synthesis by diet and plasma amino acids. *Am. J. Clin. Nutr.*, **28**, 638–647.

*(Received February 16, 1976.*
*Revised March 24, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 121–126

# INFLUENCE OF DOPAMINE AND NORADRENALINE ON ISOLATED CEREBRAL ARTERIES OF THE DOG

N. TODA
Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan

1 Effects of dopamine and noradrenaline were compared in helically-cut strips of canine cerebral arteries.

2 Dopamine caused a greater maximal contraction than noradrenaline, although the $ED_{50}$ for noradrenaline was appreciably less. The contraction induced by these amines was reversed to a relaxation by treatment with phenoxybenzamine.

3 Relaxation induced by dopamine in phenoxybenzamine-treated and prostaglandin-contracted cerebral arteries was not influenced by 1 µM propranolol, while relaxation induced by noradrenaline at low concentrations (2 µM and 10 µM) was significantly attenuated. Neither aminophylline nor atropine affected the relaxant effect of dopamine.

4 A mechanism other than $\beta$-adrenergic, cholinergic or adenosine-related appears to be involved in the relaxation elicited by dopamine in cerebral arterial strips.

## Introduction

Specific dopamine receptors have been postulated to exist in renal, mesenteric and coronary vessels from the following findings; in anaesthetized dogs, dopamine causes vasodilatation which is not blocked by $\beta$-adrenoceptor blocking agents but by haloperidol, bulbocapnine, phenothiazines and apomorphine (reviewed by Goldberg, 1972) and this specific action of dopamine is observed only in renal (McDonald, Goldberg, McNay & Tuttle, 1963; McNay, McDonald & Goldberg, 1965), mesenteric (Eble, 1964) and coronary (Schuelke, Mark, Schmid & Eckstein, 1971) but not in femoral and carotid vasculatures. Recent studies in our laboratory have demonstrated that dopamine causes a dose-related relaxation in canine isolated renal, mesenteric and coronary arteries treated with phenoxybenzamine and this relaxation is not influenced by propranolol (Toda & Goldberg, 1973; 1975; Goldberg & Toda, 1975).

It has been reported that continuous infusion of dopamine via the femoral vein increases the cerebral blood flow of anaesthetized dogs after treatment with $\alpha$-adrenoceptor blocking agents; the increase is not inhibited by propranolol but by pimozide (von Essen, 1974), a dopamine antagonist (Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970). However, when such experiments are done *in situ*, the possible involvement of regulatory factors in cerebral circulation and the interaction of these factors with actions of dopamine cannot be completely excluded.

The present study was therefore undertaken to elucidate the direct action of dopamine on cerebral arteries isolated from dogs, and to make a comparison with the action of noradrenaline.

## Methods

Mongrel dogs of either sex, weighing 7 to 15 kg, were anaesthetized with intraperitoneal injections of sodium pentobarbitone (50 mg/kg) and killed by bleeding from the common carotid arteries. The brain was isolated, and basilar and middle cerebral arteries (0.4 to 0.8 mm outside diameter) were rapidly removed. The arteries were cut helically at an angle of approximately 45° into strips 20–25 mm in length. The helical strips were fixed vertically between hooks in a 20 ml muscle bath containing the nutrient solution. Hooks anchoring the upper end of the strips were connected to the lever of a force-displacement transducer (Nihonkoden Kogyo Co., Tokyo, Japan), and the resting tension was adjusted to 1.5 g which has been found to be optimal for producing the maximum contraction (Toda, Hayashi & Hatano, 1976). The bathing medium was maintained at $37 \pm 0.5$°C and aerated with a mixture of 95% $O_2$ and 5% $CO_2$. The composition of the solution was as follows (mM): $Na^+$, 162.1; $K^+$, 5.4; $Ca^{++}$, 2.2; $Cl^-$, 157.0; $HCO_3^-$, 14.9; and dextrose, 5.6. Before the start of experiments, the preparations were allowed to equilibrate for 90 to 120 min in the bathing medium, during which time the fluid was replaced every 15 to 20 minutes.

The contractile response to 30 mM $K^+$ was first obtained and then the preparations were repeatedly washed. The dose-contraction relationship to dopamine or noradrenaline was then obtained and the values of contractions induced by these amines relative to the $K^+$-induced contraction are presented in the text and figures. In order to minimize the response to stimulation of $\alpha$-adrenoceptors, preparations were treated for 60 min with 10 µM phenoxybenzamine. The phenoxybenzamine-containing solution was then replaced with normal solution in which the preparations were allowed to equilibrate for 30 to 40 minutes. Arterial strips were always contracted with prostaglandin $F_{2\alpha}$ for demonstration of the relaxant effects. After the dose-relaxation relationship of the amines had been obtained, papaverine in a concentration of 100 µM was added to obtain maximum relaxation (Toda, 1974). Values of the relaxation induced by the amines relative to the papaverine-induced relaxation are presented in the text and figures. Dopamine or noradrenaline was added directly to the bathing medium in cumulative concentrations. Preparations were treated for 20 min with blocking agents before the addition of dopamine or noradrenaline. Contractions and relaxations were displayed on an ink-writing oscillograph (Sanei Sokki Co., Tokyo, Japan). Results shown in the text, figures and table are expressed as mean values ± standard errors of the means. Statistical analyses were made using Student's *t* test.

Drugs used were dopamine hydrochloride, (±)-noradrenaline hydrochloride, phenoxybenzamine hydrochloride, phentolamine mesylate, (±)-propranolol hydrochloride, sotalol hydrochloride, aminophylline, atropine sulphate, adenosine, papaverine and prostaglandin $F_{2\alpha}$ (Ono Co.).

## Results

### *Contraction induced by dopamine and noradrenaline*

The addition of dopamine in concentrations ranging from 0.2 µM to 500 µM caused a dose-related contraction in canine isolated cerebral arteries. The maximum contraction induced by dopamine (458 ± 69 mg) was approximately double the contraction induced by 50 µM noradrenaline (270 ± 59 mg) (Figure 1). In all six strips in which the effect of dopamine and noradrenaline was compared, the dopamine-induced contraction was always greater. The addition of noradrenaline in a concentration of 50 µM elicited a slight, transient contraction followed by a sustained relaxation, and when the concentration was increased to 200 µM, a marked relaxation always occurred. The average $ED_{50}$ of dopamine was approximately 100 times the value of noradrenaline (Table 1).

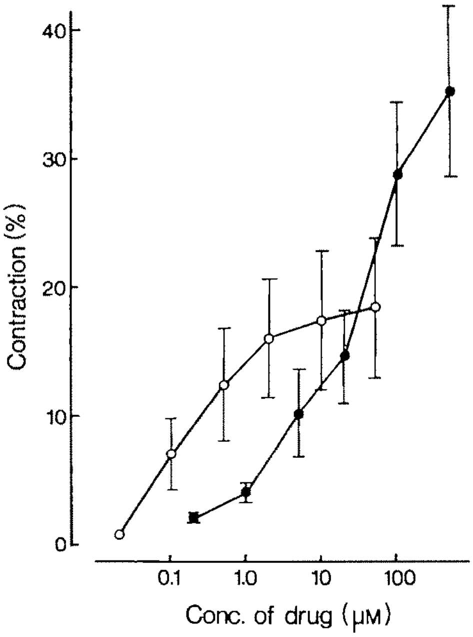


**Figure 1** Dose-response curve to dopamine (●) and noradrenaline (○) in canine isolated cerebral arteries. Ordinate scale, mean values of contractions relative to the $K^+$ (30 mM)-induced contraction; mean absolute contractions induced by $K^+$ in experiments with dopamine and noradrenaline were 1424 ± 142 mg and 1382 ± 197 mg, respectively. Vertical bars represent s.e. means. The dopamine responses are the mean results from 22 preparations; those for noradrenaline are the mean results from 10 preparations.

The contractile response of cerebral arterial strips to dopamine and noradrenaline was reversed to a relaxation following treatment with phenoxybenzamine or phentolamine. Typical recordings of the effects of dopamine and noradrenaline before and

**Table 1** Median effective concentrations of dopamine and noradrenaline in isolated cerebral arteries of the dog

| *Response* | *Median effective concentrations (µM)* | |
|---|---|---|
| | *Dopamine* | *Noradrenaline* |
| Contraction | 51 ± 7.4 (19) | 0.42 ± 0.1 (10) |
| Relaxation | 31 ± 9.1 (20) | 79 ± 4.4 (10) |

Figures in parentheses indicate the number of preparations used.

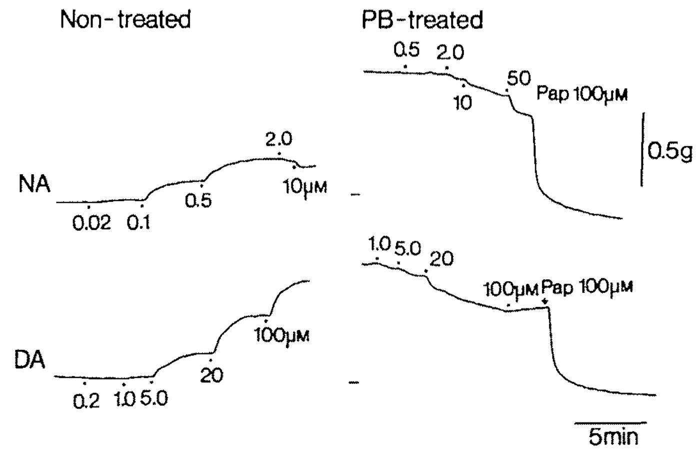


**Figure 2** Responses of a basilar arterial strip to dopamine and noradrenaline. After the response to 30 mM $K^+$ (2.32 g contraction) was obtained and the preparation was repeatedly washed, dose-response relationships to noradrenaline and dopamine were obtained. The preparation was then treated for 60 min with 10 µM phenoxybenzamine (PB), washed and equilibrated in normal solution. When the prostaglandin (1 µM)-induced contraction levelled off, the dose-response relationship for noradrenaline was obtained and (100 µM) papaverine (Pap) was added. After repeated washing, the preparation was again treated for 20 min with 10 µM phenoxybenzamine and washed. The strip was contracted with 1 µM prostaglandin $F_{2\alpha}$ and the dose-relationship for dopamine was obtained. Horizontal lines just left of the right tracings represent the level prior to the addition of prostaglandin. Drug concentrations= µM.

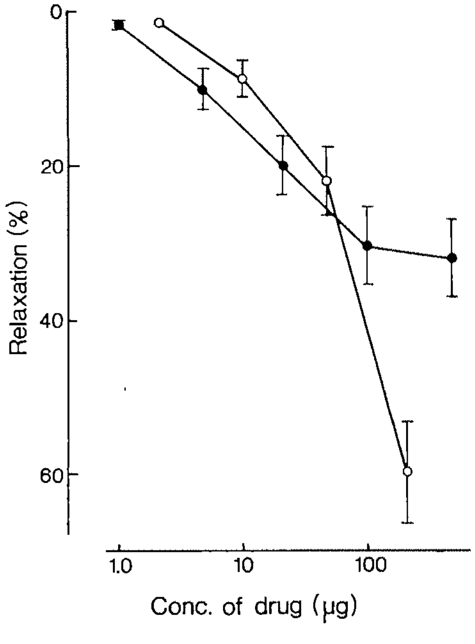


**Figure 3** Dose-response curves to dopamine (●) and noradrenaline (O) in isolated cerebral arteries. Ordinate scale, mean values of relaxations relative to the papaverine (100 µM)-induced relaxation; mean absolute relaxations by papaverine in experiments with dopamine and noradrenaline were 694 ± 63 mg and 518 ± 94 mg, respectively. Average contractions induced by prostaglandin in experiments with dopamine and noradrenaline were 398 ± 38 mg and 322 ± 66 mg, respectively. Dopamine responses are mean results from 22 preparations, those for noradrenaline are the mean results from 14 preparations.

after treatment with phenoxybenzamine in a canine basilar artery are presented in Figure 2.

### *Relaxation induced by dopamine and noradrenaline*

After treatment with phenoxybenzamine, the preparations were contracted with prostaglandin $F_{2\alpha}$ (0.1 to 3 µM) for the determination of dose-relaxation relationships. The results with dopamine and noradrenaline are summarized in Figure 3. The maximum relaxation attained with 200 µM noradrenaline was considerably greater than that with 500 µM dopamine. The mean value of the $ED_{50}$ for dopamine was approximately one third the value for noradrenaline (Table 1).

In nine cerebral arterial strips treated with phenoxybenzamine and then washed, the dose-response curve to dopamine was not significantly altered by treatement for 20 min with 1 µM propranolol (Figure 4) or by 10 µM sotalol. On the other hand, the relaxation induced at low concentrations of noradrenaline (2 µM and 10 µM) was

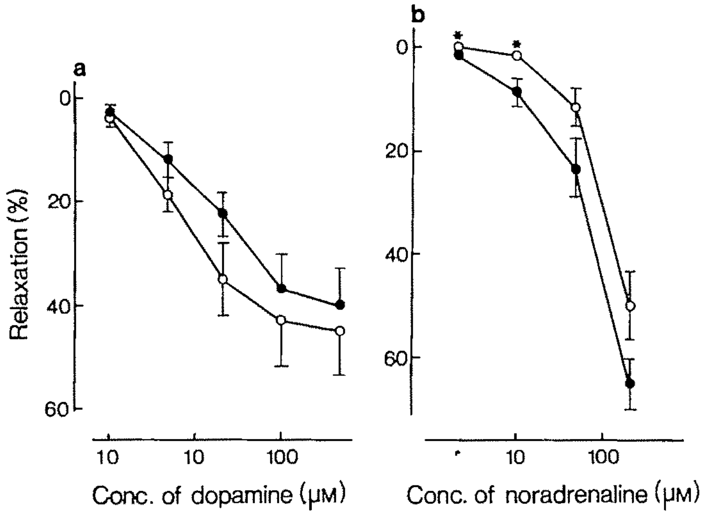


**Figure 4** Modification by propranolol (●) of the relaxant effect of (a) dopamine and (b) noradrenaline in cerebral arterial strips. Ordinate scales, mean values of relaxations relative to papaverine (100 μM)-induced relaxation; mean absolute relaxations induced by papaverine in control (●) and propranolol-treated (O) preparations were 792±80 mg and 742±124 mg, respectively (a), and 390±71 mg and 374±75 mg, respectively (b). Average contractions induced by prostaglandin in control and propranolol-treated preparations were 408±55 mg and 422±52 mg, respectively (a) and 272±60 mg and 278±54 mg, respectively (b). In (a) 9 preparations were used for both control and propranolol-treated results; in (b) 10 preparations were used in each case. *, Significantly different from respective controls, $P<0.05$.

significantly attenuated by the same concentration of propranolol.

The relaxant effect of dopamine was also unaffected by aminophylline (20 μM), which significantly reduced the relaxation induced by 1 μM adenosine from 34.7±5.2% to 15.8±3.6% (values relative to papaverine-induced relaxation) ($n=10$, $P<0.01$). Atropine (1 μM) did not reduce the relaxation in response to dopamine.

## Discussion

In isolated cerebral arteries of the dog, the addition of dopamine caused a contraction, the maximum contraction and the $ED_{50}$ being greater than those seen with noradrenaline. In anaesthetized dogs, the major effect of dopamine intravenously applied is a vasoconstriction (von Essen, 1974). Such vascular

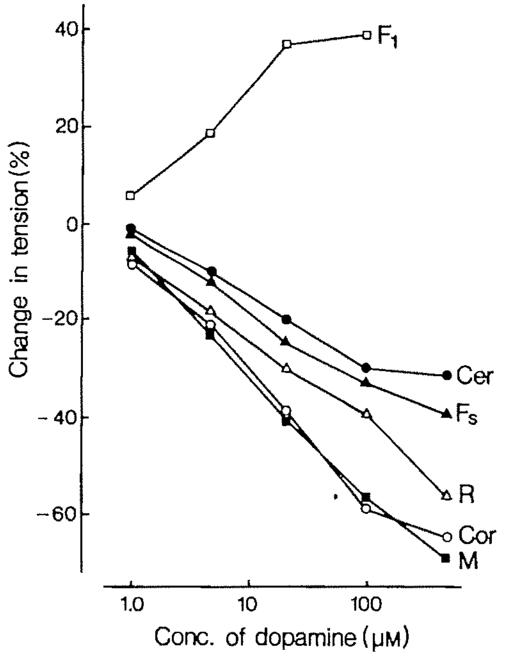


**Figure 5** Comparisons of the relaxant effect of dopamine in different arteries from dogs. Ordinate scale, mean values of relaxations relative to the papaverine-induced relaxation except in large femoral arteries ($F_1$) in which the mean contraction relative to the contraction induced by prostaglandin added to contract the arteries prior to dopamine are presented. Data for mesenteric (M), renal (R), small femoral ($F_s$) and large femoral arteries (Goldberg & Toda, 1975) and those for coronary arteries (Cor) (Toda & Goldberg, 1975) have been published elsewhere. Cer, cerebral arteries. Preparations were treated with 10 μM phenoxybenzamine.

contraction observed *in vitro* and *in vivo* was reversed to a relaxation following treatment with $\alpha$-adrenoceptor blocking agents, thus the contractile response is produced by the stimulation of $\alpha$-adrenoceptors as is the case with noradrenaline. However, in isolated cerebral arteries, dopamine elicited a greater contraction than did noradrenaline, while the latter elicits a greater contraction in canine isolated renal, mesenteric and femoral arteries (unpublished data). Two possibilities might account for such a discrepancy: a greater affinity of dopamine for $\alpha$-adrenoceptors in cerebral arteries and a greater relaxant effect of noradrenaline at high concentrations. When concentrations of noradrenaline were raised to 50 µM or higher, marked relaxation occurred. Further, in coronary arteries in which $\beta$-adrenoceptors are predominant, noradrenaline causes a relaxation, while dopamine causes a contraction (Toda & Goldberg, 1975). These findings suggest that the latter alternative is more likely.

Relaxation induced by dopamine in phenoxybenzamine-treated and prostaglandin-contracted cerebral arteries was not significantly influenced by propranolol, atropine or aminophylline at concentrations sufficient to attenuate the relaxant effect of noradrenaline, acetylcholine or adenosine, respectively. These findings support the hypothesis that $\beta$-adrenergic, cholinergic and adenosine-related mechanisms are not involved in the genesis of dopamine-induced relaxation in cerebral arteries as is the case in coronary arteries (Toda, Hojo, Sakae & Usui, 1975). The vasodilatory response of the cerebral circulation of anaesthetized dogs to dopamine is abolished by pimozide (von Essen, 1974), while the relaxant effect of dopamine in isolated arteries, including renal, mesenteric, coronary and cerebral, is not antagonized by dopamine antagonists, haloperidol, chlorpromazine, apomorphine and bulbocapnine, since these drugs applied at high concentrations produce marked relaxation in isolated arteries (Toda & Goldberg, 1975; Goldberg & Toda, 1975).

Regional differences in the response of isolated canine arteries of the dog to vasoconstrictor and dilator agents have been demonstrated (Bohr, Goulet & Taquini, 1961; Toda & Fujita, 1973; Toda, 1974). The relaxant effect of dopamine in different arteries under the same experimental conditions are summarized in Figure 5. The potency of dopamine in causing a relaxation is in the order, mesenteric = coronary > renal > small femoral > cerebral. Large femoral arteries contract in response to dopamine even after treatment with phenoxybenzamine. Relaxation caused by stimulation of specific receptors such as $\beta$-adrenoceptors and cholinoceptors, is appreciably less in cerebral arteries as compared with the relaxation observed in peripheral arteries (Toda, 1974), and the same is true in the case of dopamine, while non-specific relaxation induced by papaverine is not markedly different in cerebral and peripheral arteries.

Thanks are due to Miss M. Ohara for assistance with the manuscript and to Mr H. Usui for technical assistance.

## References

ANDÉN, N.E., BUTCHER, S.G., CORRODI, H., FUXE, K. & UNGERSTEDT, U. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmac.*, **11**, 303–314.

BOHR, D.F., GOULET, P.L. & TAQUINI, A.C. (1961). Direct tension recording from smooth muscle of resistance vessels from various organs. *Angiology*, **12**, 478–485.

EBLE, J.N. (1964). A proposed mechanism for the depressor effect of dopamine in the anaesthetized dog. *J. Pharmac. exp. Ther.*, **145**, 64–70.

GOLDBERG, L.I. (1972). Cardiovascular and renal actions of dopamine: potential clinical applications. *Pharmac. Rev.*, **24**, 1–29.

GOLDBERG, L.I. & TODA, N. (1975). Dopamine induced relaxation of isolated canine renal, mesenteric and femoral arteries contracted with prostaglandin $F_{2\alpha}$. *Circulation Res.*, **36** and **37** (Suppl.), I–97–I–102.

McDONALD, R.H., GOLDBERG, L.I., McNAY, J.L. & TUTTLE, E.P. (1963). Augmentation of sodium excretion and blood flow by dopamine in man. *Clinical Res.*, **11**, 248.

McNAY, J.L., McDONALD, R.H. & GOLDBERG, L.I. (1965). Direct renal vasodilatation produced by dopamine in the dog. *Circulation Res.*, **16**, 510–517.

SCHUELKE, D.M., MARK, A.L., SCHMID, P.G. & ECKSTEIN, J.W. (1971). Coronary vasodilatation produced by dopamine after adrenergic blockade. *J. Pharmac. exp. Ther.*, **176**, 320–327.

TODA, N. (1974). The action of vasodilating drugs on isolated basilar, coronary and mesenteric arteries of the dog. *J. Pharmac. exp. Ther.*, **191**, 139–146.

TODA, N. & FUJITA, Y. (1973). Responsiveness of isolated cerebral and peripheral arteries to serotonin, norepinephrine, and transmural electrical stimulation. *Circulation Res.*, **33**, 98–104.

TODA, N. & GOLDBERG, L.I. (1973). Dopamine-induced relaxation of isolated arterial strips. *J. Pharm. Pharmac.*, **25**, 587–589.

TODA, N. & GOLDBERG, L.I. (1975). Effects of dopamine on isolated canine coronary arteries. *Cardiovascular Res.*, **9**, 384–389.

TODA, N., HAYASHI, S. & HATANO, Y. (1976). Length-tension relationship in isolated canine cerebral arteries. *Jap. J. Pharmac.*, **26**, 126–128.

*Br. J. Pharmac.* (1976), **58**, 127–135

# THE ACTIONS OF NATURAL SECRETIN ON THE SMALL INTESTINAL VASCULATURE OF THE ANAESTHETIZED CAT

P.D.I. RICHARDSON

Department of Physiology, The Medical College of St. Bartholomew's Hospital,
Charterhouse Square, London EC1M 6BQ

1 A plethysmographic preparation of cat jejunum was used to measure changes in tissue volume and capillary filtration coefficient (CFC), simultaneously with measurements of arterial and venous pressures, and total blood flow.

2 Secretin was infused and injected intravenously and also infused intra-arterially in relatively small doses. Probable resulting blood concentrations were compared with those determined under physiological conditions in other investigations.

3 By intravenous or intra-arterial infusion, secretin caused increases in CFC, indicating an increased functional exchange vessel area, and increases in jejunal volume, indicating increased vascular capacitance. The jejunal blood flow increased whilst the blood pressure remained essentially unchanged.

4 By intravenous injection, secretin caused rises in jejunal volume and reductions in calculated jejunal vascular resistance over the same dose range. Effects were statistically significant at 500 mu/kg and higher doses caused reductions in systemic arterial pressure.

5 The calculated peak blood concentrations of secretin resulting from the lower doses used in this investigation were of the same order of magnitude as those determined under physiological conditions in man.

6 It is possible that at physiological concentrations secretin causes an increased functional exchange vessel area in the small intestine, and may also increase the total blood flow through this tissue.

## Introduction

The first report of the physiological and pharmacological properties of an extract of mammalian duodenum and jejunum showed that the active principle in the extract, secretin, caused a reduction in arterial blood pressure when injected intravenously, as well as stimulating pancreatic secretion (Bayliss & Starling, 1902). This early report of the vasodepressor action of this hormone has been extended subsequently by Ross (1970) and Fasth, Filipsson, Hulten & Martinson (1972) who showed that large doses of secretin injected intra-arterially or intravenously to the anaesthetized cat caused increases in superior mesenteric arterial blood flow.

Plethysmographic preparations of the cat small intestine have been used previously for the simultaneous measurement of blood flow, changes in vascular capacitance and capillary filtration coefficient (CFC) by Folkow, Lundgren & Wallentin (1963), and these techniques have been evaluated by Mellander & Johansson (1968) and Folkow & Mellander (1970). It has previously been demonstrated that changes in CFC may be evoked by vasoactive substances adminstered in doses inadequate to affect the recorded intravascular pressures or the total blood flow through a tissue (Folkow *et al.*, 1963; Richardson, 1974, 1975a, 1975b).

Secretin is present in mammalian jejunum in large quantities (Bayliss & Starling, 1902; Bloom, 1974) and relies for its main effect of stimulating pancreatic exocrine secretion on its release into the systemic circulation, thereby reaching its target organ. It is therefore possible that the vascular effects of secretin on the small intestine might be due to the hormone gaining access through the systemic circulation, as well as by local release. For this reason, in the present investigation emphasis has been placed on the effects of secretin injected and infused intravenously to animals in which both the sympathetic innervation to the small intestine and the suprarenal glands have been preserved. In this way, the pattern of responses of the small intestinal vasculature to elevated systemic blood levels of secretin has been established, and this has been compared with the effects of intra-arterial infusions of secretin on this vascular bed.

A preliminary report of part of the results of this investigation has been presented to the British Pharmacological Society (Richardson, 1976).

## Methods

The preparations used were similar to those described previously for the measurement of blood flow, changes in tissue volume and capillary filtration coefficient (CFC) in the small intestine (Folkow *et al.*, 1963; Richardson, 1974). Eleven healthy cats of either sex, weighing between 2.40 and 3.87 kg (3.02 ± 0.40, mean ± s.d., kg) were deprived of food for 24 h but allowed access to water *ad libitum* before the induction of anaesthesia with halothane. The right saphenous vein was cannulated, and anaesthesia maintained by an intravenous injection of chloralose (Kuhlmann, Paris; 70 mg/kg).

Following a midline laparotomy, the spleen, omenta, large intestine, duodenum, pancreas and distal small intestine were extirpated, leaving a loop of proximal jejunum weighing 56.5 ± 17.2 g (mean ± s.d.). The animals were treated with heparin (Weddel Pharmaceuticals; 250 i.u./kg, i.v., followed by 100 i.u./kg i.v., hourly) and the superior mesenteric vein cannulated. Blood from the superior mesenteric vein was continuously returned to the animal via the cannulated right external jugular vein, using a Watson-Marlow MHRE-200 roller pump (Richardson, 1974).

In most experiments, the superior mesenteric artery and its accompanying sympathetic periarterial nerves were carefully preserved, but in some ('isolated') preparations, the sympathetic periarterial nerves were divided and the superior mesenteric artery cannulated and perfused from a cannulated common carotid artery.

The prepared loop of small intestine was inserted into a rhomboid-shaped Perspex plethysmograph, which was then sealed and connected to a recorder to measure changes in the volume of the tissue (Richardson, 1974). The temperature of the contents of the plethysmograph, and of the animal, was maintained at 37–38°C with table heaters and radiant lamps. All animals breathed spontaneously throughout the experiments, and the volume of the blood localized in the external circuit was compensated for by an intravenous injection of a corresponding volume of low molecular weight dextran in normal saline (Rheomacrodex, Pharmacia) immediately before cannulation of the superior mesenteric vein.

### Measurements

*Systemic arterial blood pressure* was measured from a cannulated femoral artery by means of a Statham P23Gb strain-gauge transducer. Phasic pressure was recorded, and mean pressure either derived as diastolic + $\frac{1}{3}$ pulse pressure, a derivation which gave values in agreement with those obtained by switching the phasic waveform through an averaging circuit with a time-constant of about 3 s, or obtained by passing the phasic signal through a Devices (Model 3502) averaging circuit with selectable time constants of 0.5, 1 and 2 seconds. In this case, both mean and phasic pressures were displayed continuously.

*Arterial perfusion pressure* was measured in some experiments in addition to the systemic arterial blood pressure. A small branch of the superior mesenteric artery was cannulated and connected to a Consolidated Electrodynamics (Model L212) strain-gauge transducer: electronically averaged mean pressure was recorded continuously.

In the 'isolated' preparations, mean arterial perfusion pressure was measured with the same system from a 'T'-piece in the cannula, close to the point of cannulation of the superior mesenteric artery.

*Heart rate* was measured with a Devices (Model 4521) ratemeter triggered from the systemic arterial pressure waveform. The calibration was checked by running the recorder at high paper speed and counting the heart rate directly from the arterial pressure waveform.

*Superior mesenteric venous pressure* was measured just outside the plethysmograph from a 'T'-piece in the superior mesenteric venous cannula, with a Statham P23V or P23Ia strain-gauge transducer. The outlet from the superior mesenteric vein was adjusted so that under control conditions the venous pressure was about +3 mmHg relative to the level of fluid in the plethysmograph so that the veins within the plethysmograph were patent, thereby preventing an increase in the postcapillary resistance.

All pressure transducers were calibrated with mercury or water manometers immediately before each experiment: zero reference pressures were taken *post mortem* as those recorded with the catheter tips open to air *in situ*.

*Blood flow.* In all experiments, the superior mesenteric venous flow was recorded continuously with a cannulating flow probe in the superior mesenteric venous cannula, and an electromagnetic flowmeter (Cardiovascular Instruments). Mean blood flow was obtained by passing the phasic waveform through an averaging circuit with a time constant of 0.6 second.

In the isolated preparations, phasic and mean arterial inflow were also recorded, by means of a second cannulating flow probe and electromagnetic flowmeter.

All flow probes were calibrated *in situ* with whole blood; zero positions were established by temporary occlusion of the cannulae downstream from the flow probes.

*Changes in jejunal volume* were recorded as described previously (Richardson, 1974), by a displacement technique, measuring the upthrust of fluid in continuity with the plethysmograph contents upon the sensor of an isometric transducer, Devices (Model 4151).

After appropriate amplification, all variables were displayed continuously on a Devices (Model M-19) rectilinear recorder.

### *Calculations*

*Jejunal vascular resistance* was calculated as (mean arterial pressure — mean superior mesenteric venous pressure)/mean blood flow, and expressed as mmHg ml$^{-1}$ min 100 g.

*Capillary filtration coefficient* was measured essentially as described by Folkow *et al.* (1963): superior mesenteric venous pressure (SMVP) was elevated by 10 cmH$_2$O for periods of 1 minute. This caused a characteristic increase in the volume of the tissue within the plethysmograph (Figure 1): the volume increases were biphasic, the first phase being due to the passive distension of the capacitance vessels, and the second, slow and continuous phase being due to the transudation of fluid from exchange vessels into perivascular spaces (Folkow *et al.*, 1963; Mellander & Johansson, 1968; Johnson & Richardson, 1974).

The slope of this second phase, expressed as ml of fluid transuded per minute, per mmHg rise in SMVP, per 100 g of tissue (ml min$^{-1}$ mmHg$^{-1}$ 100 g$^{-1}$) is the capillary filtration coefficient (CFC).

### *Drug administration*

Hexamethonium bromide (BDH), propranolol hydrochloride (Inderal, ICI) and natural secretin (Boots) were used in this study. Doses of secretin are expressed in Crick-Harper-Raper Units (u) or milliunits (mu). One unit is equivalent to 4 clinical units and also equivalent to 62.5 ng (manufacturer's data).

Secretin was infused and injected intravenously, and also infused intra-arterially. Infusions were at a rate of 1.0 ml/min from a Watson-Marlow MHRE-200 pump precalibrated against a static pressure in excess of 150 mmHg to ensure a constant inflow against arterial pressures. Intravenous injections were in volumes not exceeding 0.25 ml, washed in to a total injectate volume of 1.0 ml with 0.9% w/v NaCl solution (saline).

### *Expression of results*

Except where indicated to the contrary, results are expressed as means ± s.e. means; the significance of changes from control data is assessed by use of Student's *t*-test for paired data.

At least 3 and usually 4 determinations of CFC and measurements of other variables were made before and during secretin infusions, and the mean values for each set of variables, together with the percentage changes, were calculated for each experiment. Where *n* is quoted, it therefore refers to numbers of experiments and not to numbers of individual determinations of CFC and measurements of other variables. When secretin was injected, the peak effects were compared with the control values immediately preceding the injection, and percentage changes calculated from these measurements.

## **Results**

### *Control values*

Under control conditions in 7 preparations in which the superior mesenteric artery and periarterial nerves were intact, the systemic arterial mean pressure (BP) was 151.4 ± 4.2 mmHg, and the heart rate (HR) 174.6 ± 10.0 beats/minute. The superior mesenteric venous pressure (SMVP) was 3.14 ± 0.36 mmHg, at which pressures the preparations were isovolumetric under control conditons. The superior mesenteric venous blood flow (SMVF) was 55.7 ± 7.8 ml min$^{-1}$ 100 g$^{-1}$, and the calculated jejunal vascular resistance (JVR) 3.15 ± 0.57 mmHg ml$^{-1}$ min 100 g. The capillary filtration coefficient (CFC) was 0.060 ± 0.009 ml min$^{-1}$ mmHg$^{-1}$ 100 g$^{-1}$.

The control values for 4 isolated preparations fell within the ranges found in the intact preparations, the BP being 140.0 ± 4.8 mmHg, the HR 212.3 ± 13.4 beats/min, the SMVF 44.3 ± 9.3 ml min$^{-1}$ 100 g$^{-1}$, the JVR 3.63 ± 0.70 mmHg ml$^{-1}$ min 100 g, and the CFC 0.048 ± 0.004 ml min$^{-1}$ mmHg$^{-1}$ 100 g$^{-1}$, values similar to those reported previously for sympathetically-denervated preparations (Folkow *et al.*, 1963; Richardson, 1975b).

No systematic differences were observed between the mean BP and the mean arterial perfusion pressures measured either from a cannulated small branch of the superior mesenteric artery, or from a 'T' piece in the arterial cannulae in 'isolated' preparations.

### *Secretin by intravenous infusion*

Secretin was infused intravenously for 15 min periods at doses of 100 and 500 mu kg$^{-1}$ min$^{-1}$ which were selected on the basis of results from preliminary experiments; the effects are summarized in Table 1.

When secretin was infused at a rate of 100 mu kg$^{-1}$ min$^{-1}$, there were no consistent changes in BP, HR, SMVF or calculated JVR ($P > 0.30$ for each variable), the changes in individual experiments being very small and not qualitatively consistent in the series of 5 experiments in which secretin was infused at this dose level.

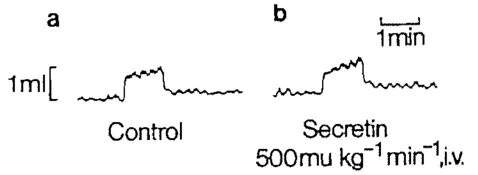


**Figure 1** Effects of an intravenous infusion of 500 mu kg$^{-1}$ min$^{-1}$ secretin on capillary filtration coefficient in the sympathetically-innervated cat jejunum. (a) under control conditions, CFC=0.035 ml min$^{-1}$ mmHg$^{-1}$ 100 g$^{-1}$; (b) during the secretin infusion, CFC=0.080 ml min$^{-1}$ mmHg$^{-1}$ 100 g$^{-1}$. When the superior mesenteric venous pressure was returned to control levels at the end of the 1 min elevation by 10 cmH$_2$O to determine the CFC, the tissue volume returned to the same value as before the elevation of venous pressure.

However, at the higher infusion of 500 mu kg$^{-1}$ min$^{-1}$ the BP fell by up to 12 mmHg, the HR rose by up to 45 beats/min and the SMVF increased by between 3.2 and 22.5 ml min$^{-1}$ 100 g$^{-1}$. In no experiment were changes detected in any of these variables which were opposite in direction to the mean changes. The effects of one such infusion of secretin in a typical experiment are illustrated in Figure 2.

At the beginning of the infusions at both dose levels, the jejunal volume increased significantly ($P<0.05$), the increase persisting throughout the infusion periods. On cessation of the infusion, the volume declined again to its pre-infusion levels.

The capillary filtration coefficient was increased at both infusion levels, the changes being statistically significant ($P<0.05$) in each case; the typical effect of elevating the SMVP by 10 cmH$_2$O for 1 min on the volume of a preparation both under control conditions and during the intravenous infusion of 500 mu kg$^{-1}$ min$^{-1}$ of secretin is shown in Figure 1.

When secretin was infused intravenously, the changes in all variables were rapid in onset, persisted for the duration of the infusions, and returned to control values within 5 min after the cessation of the infusions (Figure 2).

Since the BP fell on infusion of 500 mu kg$^{-1}$ min$^{-1}$ secretin intravenously, the possibility of a part of the effects being attributable to a baroreceptor reflex effect (Öberg, 1964) was considered: in 3 experiments the infusions were repeated after hexamethonium (5.0 mg/kg, i.v.). The effects of subsequent secretin infusions were qualitatively identical to those before hexamethonium, though quantitative comparisons are difficult because of the altered control values for all variables (Richardson, 1974).

Many of the effects of secretin on this preparation resemble those of isoprenaline (Folkow *et al.*, 1963; Richardson, 1974), and so in 3 experiments the intravenous infusion of 500 mu kg$^{-1}$ min$^{-1}$ of secretin was repeated after a dose of propranolol previously found adequate to block the effects of exogenous isoprenaline on these preparations (Richardson, 1974): the effects of secretin on the small intestinal vasculature were not appreciably modified by pretreatment with propranolol.

**Table 1** The effects of intravenous infusions of natural secretin on the small intestinal vasculature of the cat

| *Infusion rate* | *Variable (units)* | *Control* | *During infusion* | *Significance* |
|---|---|---|---|---|
| *(a) 100 mu kg$^{-1}$ min$^{-1}$* | | | | |
| | Blood pressure (mmHg) | 152.0 ± 4.7 | 152.6 ± 5.9 | NS |
| | Heart rate (beats/min) | 176.4 ± 14.0 | 180.9 ± 11.6 | NS |
| | SMVF (ml min$^{-1}$ 100 g$^{-1}$) | 54.6 ± 4.6 | 56.0 ± 5.4 | NS |
| | JVR (mmHg ml$^{-1}$ min 100 g) | 2.86 ± 0.24 | 2.81 ± 0.24 | NS |
| | Jejunal volume (ml/100 g) | | +0.27 ± 0.06 | $P<0.05$ |
| | CFC (ml min$^{-1}$ mmHg$^{-1}$ 100 g$^{-1}$) | 0.060 ± 0.009 | 0.083 ± 0.012 | $P<0.05$ |
| *(b) 500 mu kg$^{-1}$ min$^{-1}$* | | | | |
| | Blood pressure (mmHg) | 151.6 ± 4.1 | 146.2 ±4.3 | $P<0.05$ |
| | Heart rate (beats/min) | 177.4 ± 13.2 | 199.6 ±9.5 | NS |
| | SMVF (ml min$^{-1}$ 100 g$^{-1}$) | 50.8 ± 3.9 | 59.9 ±6.0 | $P<0.05$ |
| | JVR (mmHg ml$^{-1}$ min 100 g) | 3.06 ± 0.26 | 2.57 ±0.32 | $P<0.05$ |
| | Jejunal volume (ml/100 g) | | +0.83 ±0.24 | $P<0.05$ |
| | CFC (ml min$^{-1}$ mmHg$^{-1}$ 100 g$^{-1}$) | 0.060 ± 0.009 | 0.095 ±0.011 | $P<0.02$ |

All variables are expressed as means ± s.e. means; $n=5$ in all cases. The degrees of statistical significance of the differences between the mean values immediately before (control) and during the infusions of secretin are assessed using Student's *t*-test. NS=$P>0.05$; for values in Part (a), NS=$P>0.30$. SMVF=superior mesenteric venous blood flow; JVR=jejunal vascular resistance; CFC=capillary filtration coefficient. Jejunal volume=change in jejunal volume caused by the secretin infusion from control isovolumetric state.

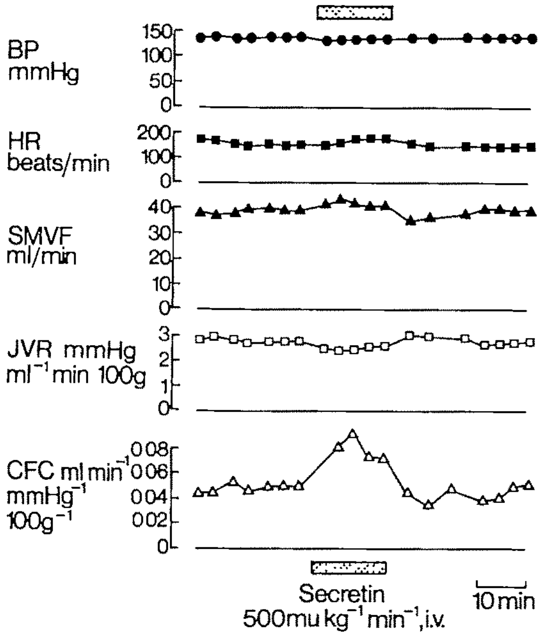


**Figure 2** The effects of an intravenous infusion of 500 mu $kg^{-1}$ $min^{-1}$ of secretin on systemic arterial mean pressure (BP), heart rate (HR), superior mesenteric venous blood flow (SMVF), the calculated jejunal vascular resistance (JVR) and the capillary filtration coefficient (CFC). Cat, female, 3.41 kg; weight of jejunum = 78 g.

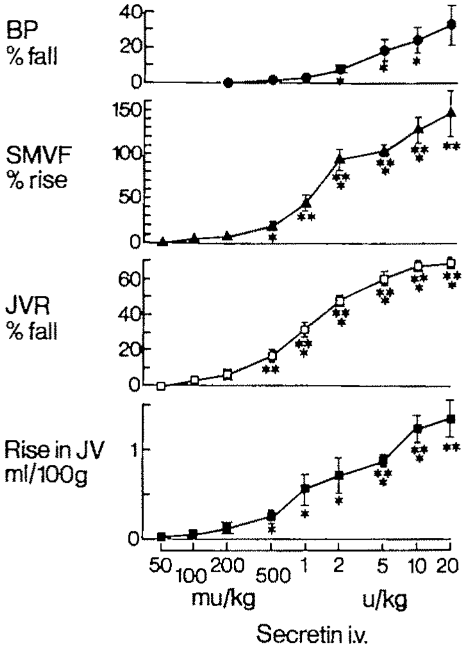


**Figure 3** Effects of increasing intravenous injections of secretin on systemic arterial mean pressure (BP; expressed as percentage reduction from pre-injection values), superior mesenteric venous blood flow (SMVF; expressed as % increases from pre-injection values), calculated jejunal vascular resistance (JVR; % reductions from pre-injection values) and the increase in jejunal volume (JV; expressed as ml/100 g of tissue). Points represent the mean of six observations and the vertical lines the s.e. means; the significance of changes from controls is shown by asterisks: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

*Secretin by intravenous injection*

Secretin was injected intravenously in increasing doses from 10 mu/kg to 20 u/kg on six occasions in order to examine the dose–response relationship of intravenous secretin on the jejunal resistance and capacitance vessels. The results are summarized in Figure 3.

The predominant effects were a transient increase in jejunal volume and a transient reduction in calculated jejunal vascular resistance: both effects occurred over the same dose range, being detectable on injection of 100 mu/kg, and being statistically significant on injection of 500 mu/kg. The reduction in calculated JVR indicates that vasodilatation occurred in the precapillary resistance vessels (Mellander & Johansson, 1968) which resulted in large increases in jejunal blood flow, although particularly at higher doses of secretin there were reductions in systemic arterial pressure (Figure 3). The heart rate rose variably, particularly at the higher doses (5–20 u/kg, i.v.) where rises of between 6 and 28 beats/min occurred in different experiments.

Although the changes in JVR and jejunal volume appear to reach maxima between 10 and 20 u/kg (Figure 3), and correlate well with those reported by Ross (1970) on intravenous injection of a single dose of 40 u/kg (= 10 clinical units/kg) secretin, no attempt was made to establish absolute maxima or to investigate the hypertensive effect which is reported to be apparent at high doses of the hormone (Ross, 1970), since the purpose of this investigation was to concentrate upon the effects of lower doses of secretin.

If the assumption is made that the whole intravenous injection of secretin was uniformly distributed throughout a blood volume of 70 ml/kg body weight, the peak blood concentration attained after injection of 100 mu/kg would have been 1.43 mu/ml (89 pg/ml) and after 500 mu/kg, 7.14 mu/ml (446 pg/ml).

*Secretin by intra-arterial infusion*

Secretin was infused intra-arterially to investigate two points: (i) whether the effects on all variables were

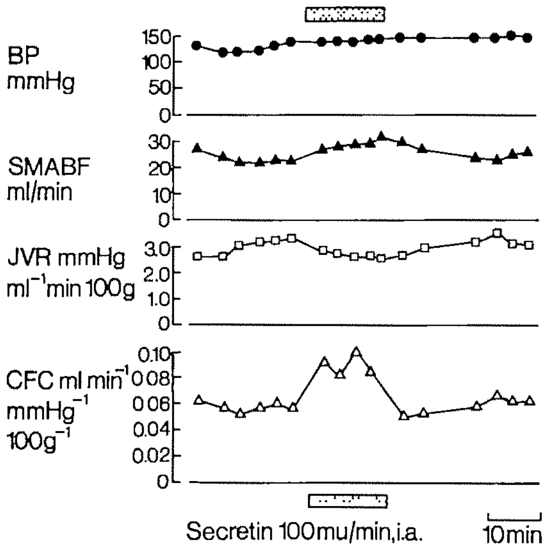


**Figure 4** Intra-arterial infusion of secretin to a 52 g loop of jejunum in a 3 kg female cat: effects are shown on systemic arterial mean pressure (BP), superior mesenteric arterial blood flow (SMABF), calculated jejunal vascular resistance (JVR) and capillary filtration coefficient (CFC). The heart rate did not change during the infusion from its control level of 241 beats/minute.

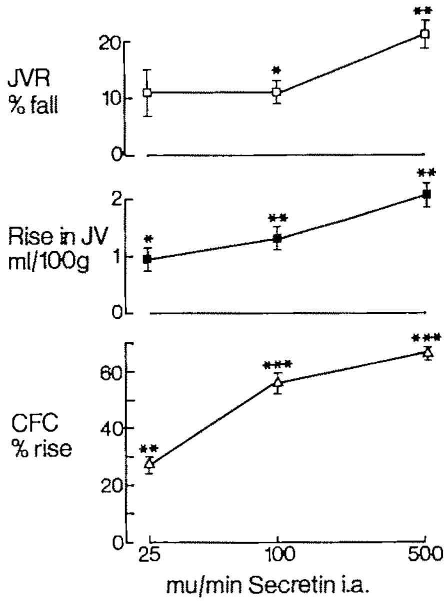


**Figure 5** The effects of intra-arterial infusions of secretin on jejunal vascular resistance (JVR; expressed as % reduction), the jejunal volume (JV; increases expressed in ml/100 g) and CFC (expressed as % increase) in four preparations. Points represent the mean values and vertical lines the s.e. means; the significance of changes from control values is shown by asterisks: $* = P < 0.02$; $** = P < 0.01$; $*** = P < 0.001$.

qualitatively and quantitatively similar to those observed on intravenous infusion, and consequently whether significant activation or deactivation of the hormone was likely to have occurred in passage through the lungs and other tissues, and (ii) whether the blood concentrations of secretin attained on intra-arterial infusion were of the same order of magnitude as those reported to occur physiologically, for if effects were produced at such concentrations, they could well be of functional significance.

Secretin was infused intra-arterially in doses of 25, 100 and 500 mu/min to each of 4 preparations in random order of doses; all infusions were of 15 min duration. The effects of a typical infusion are shown in Figure 4, and the effects summarized in Figure 5.

When secretin was infused intra-arterially, the perfusion pressure and the systemic arterial pressure remained unchanged at all dose levels employed ($P > 0.30$). The heart rate also remained unchanged at all three dose levels of secretin ($P > 0.20$). There were, however, significant increases in blood flow through the tissue which resulted in reductions in calculated jejunal vascular resistance (Figure 5), indicating that vasodilatation had occurred. In addition, there were dose-dependent increases in jejunal volume and in capillary filtration coefficient, effects which were statistically significant at all three infusion levels. The effects of intra-arterial infusions of secretin were rapid in onset, persisted for the duration of the infusions without any consistent tendency to increase or diminish, and declined quickly on cessation of infusions, the variables returning to pre-infusion levels within 5 min (Figure 4).

The mean maximum blood concentrations of secretin were assessed by dividing the amount of the hormone infused (mu/min) by the blood flow (ml/minute). For the 25 mu/min infusions, the calculated mean maximum blood concentration was 1.57 mu/ml (98 pg/ml), for the 100 mu/min infusions, 4.20 mu/ml (263 pg/ml) and for the 500 mu/min infusions, 22.19 mu/ml (1.39 ng/ml).

## Discussion

Secretin has been shown to cause increases in capillary filtration coefficient and to increase the volume of the small intestine of the anaesthetized cat, effects which were demonstrable whether the hormone was administered intravenously or intra-arterially, and which were dose-dependent and statistically significant. This extends the observation of Biber, Fara & Lundgren (1973) of the effect of intra-arterial secretin on the denervated intestine to the effects of secretin administered intravenously by injection and by infusion. In the present investigation, the effects are quantitated with respect to the administered doses. Additionally, the effects of secretin on intestinal blood flow and vascular resistance (Ross, 1970; Tibblin, 1971; Fasth *et al.,* 1972) have been confirmed, extended to the effects of much lower doses of the hormone, and the dose-response relationships established.

The use of the present techniques for the measurement of vascular dimensions is well established (Mellander, 1960; Folkow *et al.,* 1963; Mellander & Johansson, 1968; Folkow & Mellander, 1970). The changes in vascular resistance reported in the present investigation were the result of vasodilatation in precapillary resistance vessels, manifest as substantial increases in blood flow at constant arterial pressure, except where secretin was injected intravenously in large doses when a hypotensive effect was apparent (Figure 3).

Changes in CFC indicate changes either in activity of precapillary 'sphincters' or in vascular permeability, or both; it is not possible to differentiate these separate mechanisms *in vivo,* but the fact that there have not been previous reports of secretin increasing vascular permeability, supported by the observation that secretin caused no irreversible increases in intestinal volume at either control or elevated venous pressures (Figure 1) suggests that the predominant effect may well have been for secretin to dilate the intestinal precapillary 'sphincter' vessels rather than to increase vascular permeability.

An increase in tissue volume on administration of a vasoactive hormone does not constitute conclusive proof of a vasodilator action on the capacitance vessels, since the effect may be indirect, resulting from distension of the capacitance vessels caused by precapillary resistance vessel dilatation with a consequent increase in blood flow. As the increases in jejunal volume which were observed in the present experiments occurred over the same dose ranges as the reductions in vascular resistance (Figures 3 and 5), the underlying cause of the observed increase in intestinal volume cannot be ascribed with certainty either to a direct effect on the capacitance vessels, or to an indirect effect on the capacitance vessels resulting from precapillary vasodilatation. Nevertheless, it has been demonstrated that dilatation of the capacitance vessels does occur on administration of secretin, over the same dose range as dilatation of precapillary resistance vessels.

When any vasodilator agent is administered intravenously and results in a reduction in systemic arterial pressure, contributions to the observed changes in vascular dimensions in a single, sympathetically-innervated tissue may arise from myogenic and hydrostatic responses to the altered arterial pressure (Bayliss, 1902; Folkow, 1964), and also from baroreceptor reflex modulations in sympathetic vasoconstrictor tone (Öberg, 1964). The fact that the effects of the intra-arterial infusions of secretin, which did not cause changes in arterial pressure, were very similar to those observed on intravenous infusion of secretin at the dose levels chosen for these experiments suggests that the predominant effects seen on intravenous infusion were direct effects of the hormone on the intestinal vasculature. This view receives support from the similarity of the effects of intravenous secretin before and after hexamethonium, although quantitative comparisons are difficult because of the hypotensive effect of hexamethonium itself.

Significant reductions in arterial pressure did occur on intravenous injection of the larger doses of secretin (> 1 u/kg), and both hydrostatic and reflex modulations in vascular dimensions, as well as direct effects of the hormone, could well have contributed to the observed effects (Figure 3). Reductions in carotid sinus pressure in the cat cause an increase in jejunal vascular resistance and a reduction in jejunal volume, together with a reduction in CFC (Öberg, 1964). Since no attempt was made to elicit maximum responses to intravenously injected secretin, the apparent maxima shown in Figure 3 may not in fact represent the true maximum rises in jejunal volume or maximum reductions in JVR which could be attained in denervated preparations perfused at constant pressure, since the secretin-induced rises in JV and reductions in JVR may have been counteracted in these sympathetically-innervated preparations by baroreceptor reflex effects at the higher doses of secretin when the systemic arterial pressure fell.

It was shown by Ross (1970) that pretreatment with propranolol did not modify the reductions in vascular resistance caused by intravenous or intra-arterial secretin; this observation is confirmed in the present experiments and extended to the effects of secretin on CFC and the jejunal volume, both of which were unaffected by pretreatment with propranolol in a dose previously found adequate to block the effects of exogenous isoprenaline on this preparation (Richardson, 1974). Therefore, although the effects of secretin are superficially similar to those of isoprenaline on this preparation (Folkow *et al.,* 1963; Richardson, 1974), it is unlikely that they are the result of $\beta$-adrenoceptor stimulation.

The effects of secretin on the jejunal vascular bed are different from those of the structurally-related hormone, glucagon (Richardson, 1975a) which causes a fall in CFC which is reversible by $\alpha$-adrenoceptor blockade. Whilst this action of glucagon may be due to the release of catecholamines either from the suprarenal gland or from the intestine itself (Fasth & Hulten, 1971; Richardson, 1975a, 1975b), the possibility remains that the two hormones may have an essentially dissimilar action on the intestinal vascular smooth muscle, as they do on the hepatic parenchymal cells (Peterson, 1974).

Physiological blood concentrations of secretin do not appear to have been reported for the cat, though radioimmunoassay techniques have been employed to study plasma secretin concentrations in man (Bloom & Ogawa, 1973; Boden & Chey, 1973; Bloom, 1974). Fasting secretin levels range between about 100 and 500 pg/ml plasma: assuming an haematocrit of about 50%, these values correspond to blood concentrations of between 50 and 250 pg/ml. The presence of acid in the duodenum causes a rise in plasma secretin levels to between 200 and 600 pg/ml (Boden & Chey, 1973; Bloom & Ogawa, 1973), and starvation also leads to an increase in plasma secretin levels from about 50 pg/ml in the fed subject to about 175 pg/ml in starvation (Henry, Flanagan & Buchanan, 1975). It is therefore not unreasonable to assume that, in man, normal secretin levels may be about 25 pg/ml blood, rising on starvation to values up to 250 pg/ml, and perhaps even higher if acid is present in the duodenum.

The blood secretin levels in the present experiments when secretin was infused intra-arterially in doses of 25 and 100 mu/min were about 100 and 260 pg/ml respectively, and after intravenous injection of 100 mu/kg if the injected secretin had been distributed evenly throughout the bloodstream, the resultant blood concentration would have been about 90 pg/ml.

If the assumption is made that physiological secretin levels are similar in the cat and in man, the effects which were demonstrated at the lower doses of secretin employed in the present experiments could well be of functional significance, particularly as the effects were clear-cut, and in some cases statistically significant (Figures 3–5).

It is possible, on the basis of the present experiments, that when secretin levels are elevated under 'physiological' conditions, as when acid is present in the duodenum, and in starvation, the capillary filtration coefficient may be increased, causing an increase in the functional exchange vessel area in the small intestine, and particularly at the higher secretin levels falling within the 'physiological range', the total blood flow through the intestine may be increased, due to a reduction in intestinal vascular resistance. It is possible that such a vascular effect of secretin is important in maintaining high blood secretin levels in starvation, a state in which Henry *et al.* (1975) have suggested that the hormone may exert a lipolytic action.

This study was generously supported by the Medical Research Council. The author is pleased to acknowledge the skilful technical assistance of Miss June Pyke and Miss Dorinda Lobenhan, and the help of the Boots Company Limited in a literature search.

**References**

BAYLISS, W.M. (1902). On the local reactions of the arterial wall to changes in internal pressure. *J. Physiol. Lond.*, **28**, 220–231.

BAYLISS, W.M. & STARLING, E.H. (1902). The mechanism of pancreatic secretion. *J. Physiol. Lond.*, **28**, 325–353.

BIBER, B., FARA, J. & LUNDGREN, O. (1973). Vascular reactions in the small intestine during vasodilatation. *Acta Physiol. scand.*, **89**, 449–456.

BLOOM, S.R. (1974). Progress report: radioimmunoassay of intestinal hormones. *Gut*, **15**, 502–510.

BLOOM, S.R. & OGAWA, O. (1973). Radioimmunoassay of human peripheral plasma secretin. *J. Endocrinol.*, **58**, xxiv–xxv.

BODEN, G. & CHEY, W.Y. (1973). Preparation and specificity of antiserum to synthetic secretin and its use in a radioimmunoassay (RIA). *Endocrinology*, **92**, 1617–1624.

FASTH, S. & HULTEN, L. (1971). The effect of glucagon on intestinal motility and blood flow. *Acta physiol. scand.*, **83**, 169–173.

FASTH, S., FILIPSSON, S. HULTEN, L. & MARTINSON, J. (1972). The effect of the gastrointestinal hormones on intestinal motility and blood flow. *Experientia*, **29**, 982–984.

FOLKOW, B. (1964). Description of the myogenic hypothesis. *Circ. Res.*, Supt. to 14 & 15, I-279 to I-285.

FOLKOW, B., LUNDGREN, O. & WALLENTIN, I. (1963). Studies on the relationship between flow resistance, capillary filtration coefficient and regional blood volume in the intestine of the cat. *Acta physiol. scand.*, **57**, 270–283.

FOLKOW, B. & MELLANDER, S. (1970). Measurements of capillary filtration coefficient and its use in studies of the control of capillary exchange. In *Capillary permeability (Alfred Benzon Symposium II)*, pp. 614–623. Munksgaard, Copenhagen.

HENRY, R.W., FLANAGAN, R.W.J. & BUCHANAN, K.D. (1975). Secretin: a new role for an old hormone. *Lancet*, **ii**, 202–203.

JOHNSON, P.C. & RICHARDSON, J.R. (1974). The influence of venous pressure on filtration forces in the intestine. *Microvasc. res.*, **7**, 296–306.

MELLANDER, S. (1960). Comparative studies on the adrenergic neuro-hormonal control of resistance and capacitance blood vessels in the cat. *Acta physiol. scand.*, *Supplement* **176**, 5–86.

MELLANDER, S. & JOHANSSON, B. (1968). Control of resistance, exchange and capacitance functions in the peripheral circulation. *Pharmac. rev.*, **20**, 117–196.

ÖBERG, B. (1964). Effects of cardiovascular reflexes on net capillary fluid transfer. *Acta physiol. scand., Supplement* **229**, 5–98.

PETERSON, O. (1974). The effect of glucagon on the liver cell membrane potential. *J. Physiol. Lond.*, **239**, 647–656.

RICHARDSON, P.D.I. (1974). Drug-induced changes in capillary filtration coefficient and blood flow in the innervated small intestine of the anaesthetized cat. *Br. J. Pharmac.*, **52**, 481–498.

RICHARDSON, P.D.I. (1975a). The effects of glucagon and pentagastrin on capillary filtration coefficient in the innervated jejunum of the anaesthetized cat. *Br. J. Pharmac.*, **54**, 225P.

RICHARDSON, P.D.I. (1975b). An investigation of the mechanisms responsible for a reduction in capillary filtration coefficient in the innervated cat jejunum on intravenous infusion of histamine. *J. Pharm. Pharmac.*, **27**, 868–870.

RICHARDSON, P.D.I. (1976). The effects of intravenous secretin on the small intestinal vasculature of the cat. *Br. J. Pharmac.*, **56**, 358–359P.

ROSS, G. (1970). Cardiovascular effects of secretin. *Am. J. Physiol.*, **218**, 1166–1170.

TIBBLIN, S. (1971). De intestinala hormoneras effekt på splanknikus-cirkulationen. *Nordisk Medicin*, **85**, 121.

*(Received March 4, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 137–139

# HYPOTENSION IN RABBITS INFECTED WITH *Trypanosoma brucei*

P.F.L. BOREHAM

Department of Zoology and Applied Entomology, Imperial College of Science and Technology,
Prince Consort Road, London SW7 2AZ

I.G. WRIGHT

CSIRO, Division of Animal Health, Long Pocket Laboratories,
Private Bag No. 3, P.O., Indooropilly, Queensland, Australia 4068

1 Blood pressures and heart rates of 12 anaesthetized rabbits chronically infected with *T. brucei* were measured (average infection time 39 days (range 25–67)). The systolic BP was 31.4 ± 5.7 mmHg, the diastolic BP 25.0 ± 7.2 mmHg, and the heart rate 120.5 ± 24.2 beats/minute. Two rabbits were already hypotensive 10 days after infection. In 12 anaesthetized control rabbits, the systolic BP was 66.2 ± 7.3 mmHg (mean ± s.e.), the diastolic BP 60.2 ± 7.3 mmHg, and the heart rate 116.3 ± 15.9 beats/minute.

2 The intravenous injection of $3 \times 10^8$ disintegrated trypanosomes into infected rabbits lowered the blood pressure by 41.4 ± 22.0%. Pretreatment of two rabbits with aprotinin prior to administration of parasites prevented the fall in blood pressure.

3 Injection of $3 \times 10^8$ live trypanosomes complexed with hyperimmune sera produced a fall of 68.3 ± 38.4% in the systolic BP of normal rabbits. Disintegrated or live trypanosomes, or hyperimmune sera alone had no effect. Pretreatment of animals with aprotinin prior to administration of the immune complex abolished the fall in BP.

4 The results suggest that the profound hypotension in chronic trypanosomiasis is caused by complex formation of trypanosomes with antibody. Since it can be prevented by pretreatment with aprotinin, it is likely that activation of plasma kallikrein with a subsequent release of plasma kinins contributes to this effect.

## Introduction

Chronic infections of *Trypanosoma brucei* in the rabbit are characterized by the appearance of a succession of antigenic variants each of which stimulates the formation of its own antibody (Gray, 1962). Each rise in antibody titre corresponds to the disappearance of the homologous antigen variant. It is probable that, at each peak of parasitaemia, a reaction between the trypanosome and its variant antibody occurs which results in the removal of most of the trypanosomes from the circulation. This trypanosome/antibody complex formation has been implicated in the release of plasma kinins known to occur in trypanosomiasis (Boreham, 1968a, 1970). *In vitro* studies have shown that when trypanosomes are mixed with immune sera the resulting complexes are able to liberate kinins from a suitable substrate (Boreham & Goodwin, 1970). The mechanism of release appears to be the activation of Hageman factor by the immune complex which in turn converts prekallikrein to kallikrein, the kinin-forming enzyme (Boreham, 1968b; Boreham & Goodwin, 1970). It has been suggested that the release of kinins *in vivo* may have important pathological effects such as increasing vascular permeability (Goodwin, 1970) but to date proof of any such effect is lacking. In an attempt to provide such evidence, the effect of trypanosome complexes on blood pressure and the influence of a kallikrein inhibitor on this effect have been investigated.

## Methods

Rabbits were lightly anaesthetized with urethane (1.2 g/kg intravenously) and the blood pressure measured from the left carotid artery with a Bell and Howell electronic transducer. Test substances were administered through a cannula in the right jugular vein in 1 ml volumes and washed in with 0.2–0.4 ml sterile 0.9% w/v NaCl solution (saline). All animals were treated with heparin (1000 units/kg).

*Trypanosoma brucei* strain 427 were separated from heavily infected rat blood on DEAE cellulose columns by the method of Lanham (1968). Complexes of trypanosomes with antibody were prepared by incubating $3 \times 10^8$ separated trypanosomes with 2 ml of rabbit serum hyperimmune against *T. brucei* at 37°C for 30 min with gentle agitation. The suspension was centrifuged at 800 *g* for 5 min and the sediment washed twice in sterile phosphate buffer pH 7.4, before resuspending in buffer. Trypanosomes were disintegrated by repeated freezing and thawing.

## Results

Blood pressure and heart rates were measured in 12 control New Zealand white rabbits. The systolic pressure was $66.2 \pm 7.3$ mmHg, the diastolic pressure $60.2 \pm 7.3$ mmHg and the heart rate $116.3 \pm 15.9$ beats/minute. In 12 rabbits, chronic infection with *T. brucei* 427 (average infection time 39 days, range 25–67) was accompanied by profound hypotension. The systolic pressure was $31.4 \pm 5.7$ mmHg, the diastolic pressure $25.0 \pm 7.2$ mmHg and the heart rate $120.5 \pm 24.2$ beats/minute.

In two rabbits which had been infected for only ten days the blood pressures were 46/40 and 36/30 mmHg with heart rates of 136 and 124 beats/min respectively.

Intravenous injections of separated whole or disintegrated trypanosomes, or of trypanosomes heated at 96°C for 30 min had no significant effect on the blood pressure of normal rabbits even when the number injected was as high as $10^9$. Occasionally, a rapid transient fall of blood pressure lasting not more than 5 min and not exceeding about 15% was seen which was attributed to vascular reflexes caused by blockage of small vessels. However, when $3 \times 10^8$ disintegrated trypanosomes were injected intravenously into chronically infected rabbits a significant ($41.4 \pm 22.0$%), often biphasic fall in blood pressure occurred 3–5 min later in all ten animals. It persisted for at least 10 min (Figure 1a). In most cases the blood pressure remained low for 30 min, when the experiment was terminated.

In two chronically infected rabbits (day 31) pretreated with aprotinin 5000 iu/kg 5 min before administration of $3 \times 10^8$ disintegrated parasites, the mean fall in blood pressure was only 5.2%.

The effect of trypanosome/antibody complexes on 6 non-infected control rabbits produced a fall of $68.3 \pm 38.4$% in blood pressure (Figure 1b). The fall started 3–5 min after injection of the complexes. Three of these rabbits died within 15 min of administration of the complexes. Pretreatment of three non-infected rabbits with aprotinin completely abolished the hypotensive effect of complexes. Antiserum or live trypanosomes alone had no effect on blood pressure of non-infected rabbits.

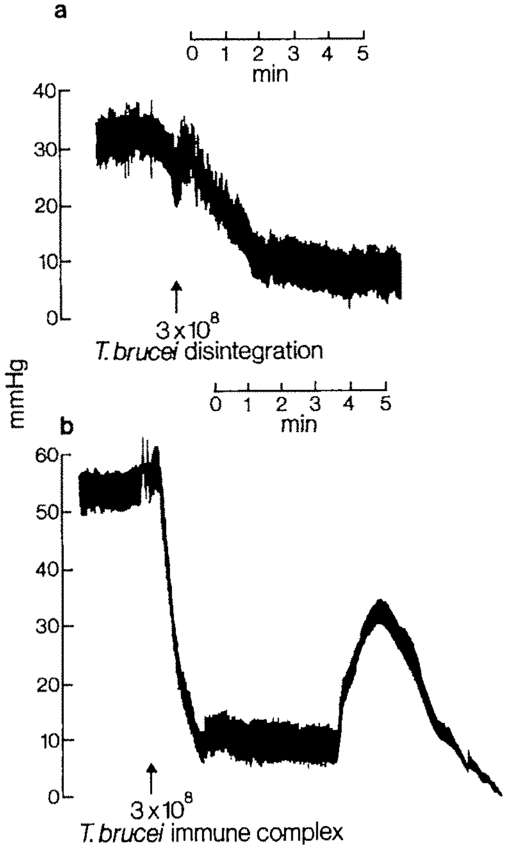


**Figure 1** (a) Effect of intravenous injection of $3 \times 10^8$ disintegrated *T. brucei* on blood pressure of an anaesthetized rabbit chronically infected with *T. brucei*. (b) Effect of intravenous injection of $3 \times 10^8$ *T. brucei* + antibody immune complex suspension on blood pressure of an anaesthetized normal rabbit.

## Discussion

Rabbits infected with trypanosomes developed severe hypotension as early as ten days after infection but showed no change in heart rate. Injections of trypanosomal antigen into chronically infected rabbits, or of immune complexes of trypanosomes and antibody into control rabbits, caused falls in blood pressure beginning 3–5 min after the injections. The falls in blood pressure were attributed to immune complexes because trypanosomes or immune sera alone had no effect on the blood pressure of non-infected rabbits. It is known that high levels of both variant and common antibodies are present as early as one week after infection (Gray, 1962). In these experiments the antigen used for intravenous injection was the same as that used to infect the rabbits, thus antibody would be present at the time of the experiment. The resulting immune complexes may produce the release of hypotensive substances from the host. The findings support earlier *in vitro* work (Boreham & Goodwin, 1970).

The fall in blood pressure in both experimental situations was inhibited by aprotinin which suggests that the hypotension was mediated by kallikrein. Aprotinin inhibits activated kallikrein but does not prevent the activation of prekallikrein or Hageman factor (Vogel & Werle, 1970). These results are consistent with the view (Boreham & Goodwin, 1970) that particulate complexes of trypanosomes with antibody adsorbed on to their surface activate Hageman factor which converts prekallikrein to kallikrein; the latter then forms kinins.

Various particles with negative charges, e.g. glass, kaolin and extracellular membranes, activate Hageman factor bound to the negatively charged surface, by a process of limited proteolysis (Wuepper & Cochrane, 1972; Cochrane, Revak & Wuepper, 1973); a variety of particulate and non-particulate immune complexes did not activate the kinin system in rabbit or human plasma, although some bacterial contaminants were able to do so (Cochrane, Wuepper, Aikino, Revak & Spiegelberg, 1972). Our results do not agree with these findings since trypanosomes alone did not cause hypotension or release kinin *in vitro* (Boreham & Goodwin, 1970).

Both kallikrein (Depot Glumorin, 4 k.u.) and bradykinin (0.2 µg) produce a fall in blood pressure of about 30% in rabbits. Thus the hypotension seen in trypanosome infections of the rabbit could be caused either by kallikrein or kinin. The present results are the first *in vivo* evidence suggesting that the release of pharmacologically active substances contributes to the pathology of trypanosomiasis.

This work was supported by a grant from the Ministry of Overseas Development to P.F.L.B. We are grateful to Sandoz Ltd. for a gift of synthetic bradykinin and Bayer Pharmaceuticals for the kallikrein.

**References**

BOREHAM, P.F.L. (1968a). Immune reactions and kinin formation in chronic trypanosomiasis. *Br. J. Pharmac. Chemother.*, **32**, 493–504.

BOREHAM, P.F.L. (1968b). *In vitro* studies on the mechanism of kinin formation by trypanosomes. *Br. J. Pharmac.*, **34**, 598–603.

BOREHAM, P.F.L. (1970). Kinin release and the immune reaction in human trypanosomiasis caused by *Trypanosoma rhodesiense. Trans. R. Soc. trop. Med. Hyg.*, **64**, 394–400.

BOREHAM, P.F.L. & GOODWIN, L.G. (1970). The release of kinins as the result of an antigen-antibody reaction. In *Bradykinin and Related Kinins: Cardiovascular, Biochemical and Neural Action, Adv. exp. Med. Biol.*, Vol. 8, ed. Sicuteri, F., Rocha e Silva, M. & Back, N., pp. 539–542. New York: Plenum Press.

COCHRANE, C.G., REVAK, S.D. & WUEPPER, K.D. (1973). Activation of Hageman factor in solid and fluid phases: a critical role of kallikrein. *J. exp. Med.*, **138**, 1564–1583.

COCHRANE, C.G., WUEPPER, K.D., AIKINO, B.S., REVAK, S.D. & SPIEGELBERG, H.L. (1972). The interaction of Hageman factor and Immune complexes. *J. clin. Invest.*, **51**, 2736–2745.

GOODWIN, L.G. (1970). The pathology of African trypanosomiasis. *Trans. R. Soc. trop. Med. Hyg.*, **64**, 797–812.

GRAY, A.R. (1962). The influence of antibody on serological variation in *Trypanosoma brucei. Ann. Trop. Med. Parsit.*, **56**, 4–13.

LANHAM, S.M. (1968). Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. *Nature, Lond.*, **218**, 1273–1274.

VOGEL, R. & WERLE, E. (1970). Kallikrein inhibitors. In *Bradykinin, Kallidin and Kallikrein, Handb. exp. Pharmac.*, N.S. Vol. 25, ed. Erdos, E.G., pp. 213–249. Berlin: Springer-Verlag.

WUEPPER, K.D. & COCHRANE, C.G. (1972). Plasma prekallikrein: isolation, characterisation and mechanism of activation. *J. exp. Med.*, **135**, 1–20.

*(Received March 9, 1976.*
*Revised April 2, 1976)*

*Br. J. Pharmac.* (1976), **58**, 141–148

# LABELLED DECAMETHONIUM IN CAT MUSCLE

R. CREESE & JENNIFER MACLAGAN[1]
Department of Physiology, St. Mary's Hospital Medical School, London W2 1PG

1 Tritium-labelled decamethonium was infused intravenously in 12 cats at final rates of 1.3–4.2 nmol $kg^{-1}$ $min^{-1}$ to produce a steady plasma concentration which ranged between 0.21–1.3 µmol/l in different experiments. Muscle contractions were elicited by nerve stimulation and the potential at the end-plate regions of superficial fibres was recorded by extracellular electrodes.

2 Under these conditions, it was not possible to obtain a steady degree of neuromuscular block. The initial decrease in muscle contractions was followed by recovery towards the original value although the concentration of decamethonium in the plasma remained constant, or in some cases rose. The initial depolarization of the end-plate region also waned during the constant infusion of the drug.

3 Once the twitch tension had returned to control values during infusion of the drug, prolongation of the infusion for a total of four hours did not produce a secondary neuromuscular block.

4 Scintillation counting showed that during infusion of labelled decamethonium the radioactivity of the muscles increased progressively with time. The uptake was less in the soleus muscle than in the fast-contracting flexor longus digitorum and extensor longus digitorum muscles. Muscles which had been denervated 12–13 days previously showed a greater uptake of labelled drug than control muscles from the contralateral limb.

5 The labelled drug was localized by autoradiography of frozen sections of leg muscles following intra-arterial injection of decamethonium. Grain counts in individual fibres showed that small amounts of decamethonium had entered the muscle fibres along their entire length, and there was increased uptake of the drug into the cell in the region of the end-plate.

6 The mechanisms underlying the waning of the pharmacological response during constant application of depolarizing drugs are discussed.

## Introduction

It has been shown by contact autoradiography that tritiated decamethonium accumulates at the end-plate region of mouse (Waser, 1966) and rat muscle (Creese & Maclagan, 1967). In the latter case, by using the more precise localization available with frozen-section autoradiography, it was found that the drug had entered the cells at the end-plate region and in adjacent parts of the fibre (Creese & Maclagan, 1970). Entry of the drug appeared to be related to the increase in membrane permeability which the drug produced and did not appear to be due to the change in membrane potential, as entry could still be demonstrated in muscles which were completely depolarized in solutions containing a high concentration of potassium (Creese & England, 1970).

The depolarizing action of decamethonium is not identical in all species. For example, in the cat, decamethonium depolarizes the end-plate region by at least 55 mV and the effect is maintained throughout the period of application of the drug (Head, 1975). Thus the potential falls below the threshold for action potential generation (Boyd & Martin, 1956) and this is probably the cause of neuromuscular blockade (Burns & Paton, 1951). In the rat, however, when decamethonium is applied, the membrane potential falls only transiently (Thesleff, 1955); consequently, prolonged depolarization is unlikely to be the cause of neuromuscular blockade in the rat.

The reasons for these species differences in the depolarizing ability of decamethonium are unknown but may reflect differences in the underlying permeability changes caused by the drug. For this reason it was of interest to know whether decamethonium enters cat muscle fibres as it does in rat muscle. In the present experiments, the uptake of tritiated decamethonium into cat hind-limb muscle fibres was measured using scintillation counting and autoradiographic methods, and the pharmacological responses of the muscle were also recorded during intravenous infusion of the drug to give a constant plasma concentration which could be maintained for several hours.

---

[1] Present address: Department of Pharmacology, Royal Free Hospital School of Medicine, Hunter Street, London WC1N 1BP.

At the same time these experiments provided an opportunity to determine whether the blocking action of the drug progressively waned during prolonged application *in vivo*. This effect is well-known in isolated muscles but has not been studied *in vivo* under conditions where the plasma concentration of the drug could be monitored to ensure a constant drug concentration.

## Methods

Cats weighing between 2 and 4 kg were anaesthetized with an intravenous injection of chloralose (80 mg/kg) and pentobarbitone sodium (4 mg/kg). A tracheal cannula was introduced so that artificial respiration could be applied when necessary. The hind limb was immobilized with steel drills through the upper and lower ends of the tibia and fibula and the tendons of attachment of either the soleus, tibialis anterior, flexor longus digitorum or extensor longus digitorum were separated from the bones and securely tied to a strain gauge (Statham 80 oz). Shielded platinum electrodes were placed on the sciatic nerve in the popliteal space and the nerve was tied central to the electrodes. Muscle twitches were elicited with supramaximal square wave stimuli of 0.1 ms duration, delivered once every 5 s, and were registered on a Sanborn recorder.

### *Recording of surface potential from tibialis anterior muscle*

The method used was that described by Burns & Paton (1951). A non-polarizable wick electrode was placed at the end-plate region of a group of surface fibres, and the location was adjusted to the point of maximal depolarization produced by a test dose of suxamethonium (30 µg/kg i.v.). A second reference electrode was placed on the patella tendon or an exposed bone. The potential between these electrodes was recorded using a stable d.c. amplifier with an input resistance of 10 MΩ and was displayed on the pen recorder. The muscle was covered with liquid paraffin at 37°C contained in a pool formed from skin flaps.

### *Administration of drugs*

Tritium-labelled decamethonium was diluted with 0.9% w/v NaCl solution (saline) and unlabelled decamethonium was added to bring the concentration to 30 µmol/l. The solution was maintained at 37°C and infused via a polythene cannula into a jugular vein by means of a Watson-Marlow peristaltic pump. The rates of infusion are given in the **Results**. On other occasions decamethonium was administered by rapid intra-arterial injection, in a small volume of saline (less than 0.2 ml) in a retrograde direction into the anterior tibial artery, while the femoral arterial flow was temporarily occluded in the thigh.

### *Blood samples*

Blood samples were obtained from a cannula in the carotid artery and collected directly into small graduated centrifuge tubes containing heparin. The cells were rapidly spun down and two aliquots of 0.1 ml of plasma were removed from each sample, added to vials containing 1.0 ml hyamine hydroxide (1 mol/l in methanol) plus 0.5 ml KOH (1 mol/l in methanol) and counted by liquid scintillation.

### *Denervation*

In five cats under pentobarbitone anaesthesia, the muscles of the left hind leg were denervated by removing 2 cm of the sciatic nerve in the popliteal space and reflecting the central stump to prevent regrowth of the nerve. The acute experiments were performed 12–14 days later.

### *Radioactive compounds and measurements*

[Methyl-$^3$H]-decamethonium dichloride with specific activity of 1.1 Ci/mmol was obtained from the Radiochemical Centre (Amersham). The compound was analyzed at Amersham by thin-layer chromatography on aluminium in chloroform-methanol (80:20, v/v) and the radiochemical purity was greater than 98%.

Hind limb muscles were rapidly dissected free from connective tissue and frozen in isopentane cooled in liquid nitrogen. The muscles were weighed in crucibles, dried under vacuum with phosphorous pentoxide and reweighed. The dried muscle was powdered and samples of 4–8 mg were added to vials which contained 1 ml hyamine hydroxide (1 mol/l in methanol). The tissues were dissolved by heating for 30 min in a water bath at 70°C and after the vials had cooled 0.5 ml KOH (1 mol/l in methanol) and 14 ml of scintillator (Creese & Taylor, 1967) were added. Background counts were measured in vials containing 1 ml hyamine solution and 0.5 ml KOH solution plus scintillator.

### *Autoradiography*

The methods used for freezing and sectioning muscles, for the preparation of the autoradiograms and for grain counting were identical to those described by Creese & Maclagan (1970).

### *Drugs*

The following drugs and chemicals were used: pentobarbitone sodium (Abbott Laboratories Ltd.); chloralose (British Drug Houses Ltd.); decamethonium dibromide (Syncurine, Burroughs Wellcome and Co., mol. wt. 418); suxamethonium chloride injection (Anectine, Burroughs Wellcome and Co.); hyamine hydroxide (1 mol/l in methanol; Nuclear Enterprises, Ltd.); heparin injection 1000 iu/ml (Paines & Byrne Ltd.).

## Results

### *Effect of decamethonium infusion on muscle contraction and end-plate depolarization*

Decamethonium (30 µmol/l) was infused into anaesthetized cats at an initial rate of between 2.1–5.2 ml/min, a range found to produce a rapid onset of neuromuscular block. As soon as the contraction height began to diminish the infusion was reduced and a final rate of 0.17–0.43 ml/min was maintained (1.3–4.2 nmol $kg^{-1}$ $min^{-1}$). This produced a transient period of steady block (approximately 90% in tibialis muscle) during which the first blood sample was taken. Figures 1, 2 and 3 show results from different experiments. The plasma concentration was subsequently steady (Figure 3), slowly decreased (Figure 1) or slowly increased (Figure 2). In all cases the muscle contractions recovered despite the continued presence of the drug.

Figure 1 shows simultaneous records of contraction and surface recording of depolarization from the tibialis anterior muscle during a 90 min infusion of decamethonium. With this extracellular recording method, *changes* in potential can be recorded but absolute values of membrane potential cannot be obtained. In this experiment the maximum depolarization of the end-plate was reached 15 min after the start of the infusion, when the plasma concentration of the drug was 0.48 µmol/l. This was associated with 99% block of the tibialis twitches. As the infusion continued, the depolarization of the end-plate waned, until after 50 min infusion the surface recording returned to the value before the infusion commenced. The muscle contractions also gradually returned to normal but their rate of recovery lagged behind the return of the potential. This may be due to the fact that these two recordings were obtained from different groups of fibres—the electrical records being restricted to a few superficial end-plates. After 70 min infusion, both mechanical and electrical recordings had returned to control values despite continued infusion of the drug. During the following 30 min infusion, the muscle contraction became larger than the initial values. Such a potentiation of the twitch has been reported previously during the period immediately following a block caused by a single intravenous injection of decamethonium (Vrbová & Maclagan, 1966; Maclagan, 1976).

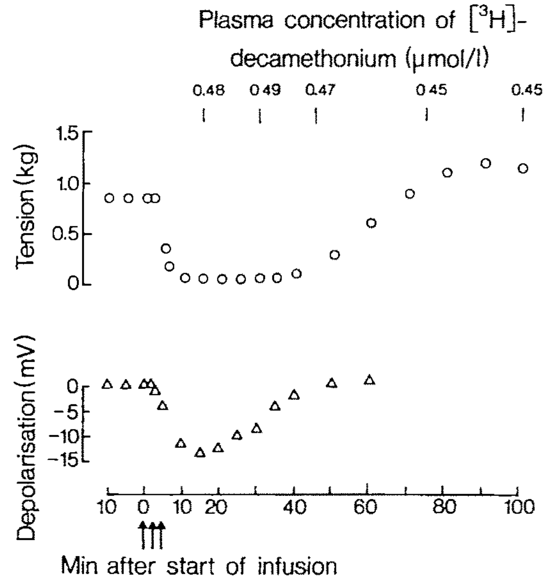


**Figure 1** Infusion of [$^3$H]-decamethonium in a cat. The graphs show simultaneous measurements of contractions of the tibialis anterior muscle elicited by supra-maximal stimulation of the motor nerve (open circles) and potential at the end-plate region of surface fibres with reference to an indifferent electrode placed on the tendon (open triangles). At the first arrow infusion of [$^3$H]-decamethonium was started at a rate of 20.4 nmol $kg^{-1}$ $min^{-1}$. The rate was adjusted at the second and third arrows and was then maintained at 4.2 nmol $kg^{-1}$ $min^{-1}$ from the third arrow until the end of the experiment. The values in the upper part of the figure indicate the plasma concentration of decamethonium in µmol/l. Time (min) is measured from the start of the infusion. Decamethonium caused initial depolarization and neuromuscular block followed by complete recovery to control values.

Similar results have also been obtained in the soleus muscle of the cat (Figure 2). This figure shows that a steady blockade was present 30 min after the start of the infusion. Two hours later the muscle twitch had returned to control values despite the continuous presence of the drug. In the case of the soleus muscle, surface recording was not possible as the scattered arrangement of the end-plates made it difficult to locate a discrete band of end-plates. The slowly contracting soleus muscle was always less sensitive to the drug than the fast contracting muscle (Jewell & Zaimis, 1951), but the time course of development of the blockade was similar in both types of muscle. This

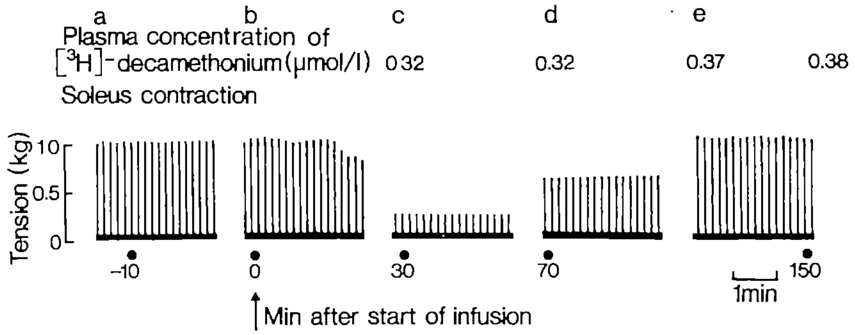


**Figure 2** Indirectly elicited maximal contractions of the soleus muscle (a) before and (b) to (e) during infusion of $[^3H]$-decamethonium. The initial infusion rate (18.2 nmol $kg^{-1}$ $min^{-1}$) was changed to 2.6 nmol $kg^{-1}$ $min^{-1}$ at 15 min and then maintained. The time in min after the start of the infusion is indicated below each section and the plasma concentration in μmol/l is given by the number above each section. Decamethonium produced a partial neuromuscular block. After 150 min infusion the block had completely recovered despite increasing plasma concentration of decamethonium.

is illustrated in Figure 3 which shows simultaneous recordings from soleus and flexor longus digitorum. In any one cat the onset of the recovery of the contraction during the infusion was very similar in both fast and slow muscle, as shown in Figure 3.

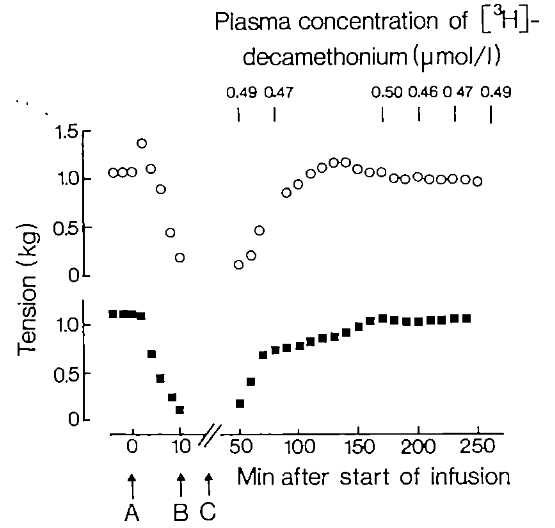


**Figure 3** Simultaneous recording of contraction of soleus (O) and flexor longus digitorum (■) during prolonged infusion of labelled decamethonium. The infusion was started at a rate of 26.7 nmol $kg^{-1}$ $min^{-1}$ at A, adjusted at B and then kept constant at 2.7 nmol $kg^{-1}$ $min^{-1}$ from C until the end of the experiment. The time scale is different after the first 10 minutes. The plasma concentration showed only small variations and the values in μmol/l are given at the top of the figure. After an initial neuromuscular block the contractions of both muscles had returned to their control values by 150 minutes. The infusion was continued for a further 100 min but there was no sign of a secondary neuromuscular block.

In four of the cats, the infusion was continued after recovery of the twitch for a total period of 4 hours. There was no sign of development of a secondary blockade with continued application of the drug, and Figure 3 gives an example of one of these experiments.

### *Uptake of $[^3H]$-decamethonium by hind limb muscles*

At various times during the infusion of decamethonium, individual muscles from the lower hind limb were rapidly dissected after ligation of the arterial branch supplying the muscle, and frozen in isopentane cooled in liquid nitrogen. The muscles were dried and powdered samples were weighed, dissolved and the radioactivity of each sample measured by liquid scintillation counting. Table 1

**Table 1** Uptake of labelled decamethonium in fast-contracting hind limb muscles of cats infused intravenously at a constant rate

| *Time in min* | *Uptake (ml/g wet muscle)* |
|---|---|
| 7– 60 | 0.15±0.021 (7) |
| 140–160 | 0.28±0.045 (7) |
| 220–260 | 0.68±0.079 (5) |

The first column gives the duration of perfusion in minutes. The uptake in leg muscles (extensor digitorum longus and flexor digitorum longus) was measured by scintillation counting and is expressed as a clearance, in ml/g. This is the ratio of the (counts per g wet muscle) to (counts per ml plasma). The limits give the s.d., with the number of muscles in parentheses. There is a progressive rise in the radioactivity found in the muscle.

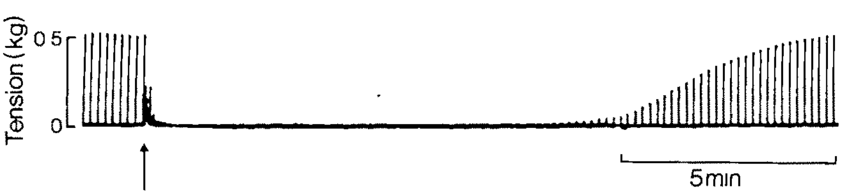


**Figure 4** Record of indirectly elicited twitches of the extensor longus digitorum muscles of the cat in response to an intra-arterial injection of [$^3$H]-decamethonium chloride (80 µg kg$^{-1}$, 0.6 mCi). Ninety minutes after the injection the muscle was rapidly frozen and prepared for autoradiography.

shows the uptake of [$^3$H]-decamethonium, expressed as a clearance in ml/g, in the fast-contracting muscles at various times after the start of the infusion. The uptake during the first hour is largely attributed to the extracellular space; thereafter, the uptake increased progressively with time to values which suggest that accumulation of the drug was occurring in the muscle fibres.

When the uptake in fast and slow muscles was compared it was found that at all stages of the infusion the uptake into the more sensitive fast-contracting muscles (extensor longus digitorum and flexor longus digitorum) was always greater than that in the slowly-contracting soleus muscle (Table 2).

Denervation of the muscles of one hind limb greatly increased the uptake compared to the unoperated muscles of the contralateral limb (see Table 3). After 140 min infusion, uptakes of over 1.0 ml/g were found in denervated muscles in some cases.

### *Autoradiography of frozen sections*

The results obtained with scintillation counting of dried muscles was consistent with an uptake of [$^3$H]-decamethonium into a compartment larger than the extracellular space. However such experiments cannot give any further indication of the location of the drug within the muscle. For this reason, autoradiography of frozen sections of muscles loaded with [$^3$H]-decamethonium was undertaken. The method has been used successfully in rat muscle (Creese & Maclagan, 1970) but in this species much greater doses of decamethonium were needed to produce neuromuscular blockade than in the more sensitive cat muscle. This is shown by the plasma concentration in the rat which ranged from 11 to 43 µmol/l, whereas in the cat a comparable degree of neuromuscular blockade is produced with a plasma concentration below 1 µmol/l. Thus in the present experiments in the cat, much lower levels of radioactivity were expected than in the previous work on rats. For this reason [$^3$H]-decamethonium dichloride was injected intra-arterially in a dose (80 µg/kg) which caused complete blockade of extensor longus digitorum for 15 min (see Figure 4). After an interval of 90 min which allowed the extracellular concentration of the drug to diminish, the

**Table 2** Uptake of labelled decamethonium in different leg muscles of cats after intravenous infusion at a constant rate.

| | | *Uptake (ml/g wet muscle)* | |
|---|---|---|---|
| *Time in min* | *Soleus* | *Extensor digitorum longus* | *Flexor digitorum longus* |
| 135 | 0.20 | – | 0.26 |
| 135 | 0.28 | – | 0.53 |
| 144 | 0.11 | 0.21 | – |
| 144 | 0.11 | 0.20 | – |
| 164 | 0.19 | 0.31 | – |
| 224 | 0.30 | 0.34 | – |
| 250 | 0.28 | 0.64 | 0.68 |
| 260 | 0.33 | – | 0.80 |
| 260 | 0.30 | 0.73 | – |

The first column gives the duration of infusion. The expression ml/g is calculated as in Table 1. In each experiment the uptake into fast-contracting extensor longus digitorum and flexor longus digitorum muscles is greater than that in the soleus muscle.

**Table 3** Effect of chronic denervation on uptake of labelled decamethonium in leg muscles of cats infused intravenously at a constant rate for 140 minutes

| *Uptake (ml/g wet muscle)* | | |
|---|---|---|
| *Control* | *Denervated* | *Muscle* |
| 0.20 | 1.22 | Soleus |
| 0.11 | 0.26 | Soleus |
| 0.11 | 0.28 | Soleus |
| 0.26 | 1.30 | Flexor longus digitorum |
| 0.21 | 0.28 | Flexor longus digitorum |
| 0.19 | 0.49 | Flexor longus digitorum |
| 0.20 | 0.32 | Extensor longus digitorum |
| 0.20 | 0.38 | Extensor longus digitorum |

The muscles were denervated 12–13 days previously. The uptake is expressed as ml/g, as in Table 1. In each case the uptake in the denervated muscle is greater than that in the control muscle taken from the other leg.

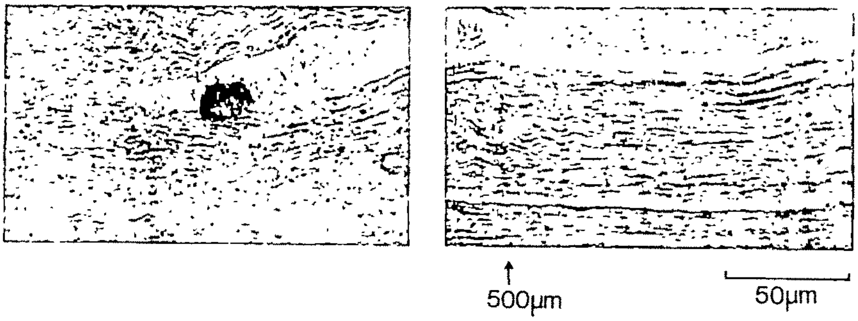


**Figure 5** Autoradiogram prepared from muscle shown in Figure 4. The grain density over the fibres is high at the end-plate (left-hand section) but considerably lower at a distance of 500 µm from the end-plate region (right-hand section).

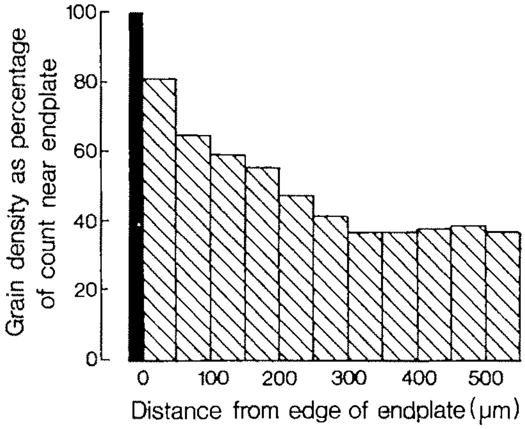


**Figure 6** Distribution of grain-density along 8 fibres from the extensor longus digitorum muscle of Figures 4 and 5. The grain density at different points along the fibre has been expressed as a percentage of the value at the end-plate region. Each column represents the mean value of eight measurements after the background count had been subtracted (s.e. mean between 6.0 and 11.5).

muscle was removed from the cat and rapidly frozen. Autoradiograms were prepared from frozen sections which were cut longitudinally and stained with an azo-dye to show the position of the esterase at the endplate region. Figure 5 shows the autoradiogram prepared from the muscle referred to in Figure 4. There is a high grain density over the fibres at the endplate region (left-hand section), but the radioactivity is much reduced at a distance of 500 µm from the end-plate (right-hand section). The resolution of the method is of the order of 1 µm (Creese & Maclagan, 1970) so these results indicate that the drug is concentrated in the cells at the end-plate region and in the surrounding parts of the fibres. The grain density between the fibres was low, indicating that the extracellular concentration of the drug had indeed been reduced to low levels 90 min after the injection.

The longitudinal distribution of radioactivity was determined by measuring the grain density along the fibres in individual fibres obtained from several sections of the same muscle. Figure 6 gives the distribution in 8 fibres from the muscle shown in Figures 4 and 5. The grain counts show that a small amount of [$^3$H]-decamethonium had entered the muscle fibres along their entire length but there was an increased uptake of the drug into the cells in the region of the end-plate.

## Discussion

In the present experiments it has been shown that prolonged infusion of [$^3$H]-decamethonium in the cat produces an initial paralysis of the indirectly elicited twitches of both fast and slow muscles, followed by recovery of the twitch response despite a plasma concentration of the drug which was maintained approximately constant. Extracellular recording of depolarization showed that this recovery of the twitch was accompanied by a waning of the end-plate depolarization produced by the drug. A similar effect has been reported in the cat tenuissimus muscle *in vivo* (Maclagan, 1962), but in the absence of measurements of plasma concentration of the drug it was attributed to a reduction in the infusion rate. These results in the cat are comparable to those obtained *in vivo* in the rat (Creese & Maclagan, 1970) and in man (Poulsen & Hougs, 1958). In all three species therefore application of a constant concentration of depolarizing drugs *in vivo* does not cause a maintained neuromuscular block.

The recovery from blockade is dose-dependent and can only be recorded within narrow dose limits. At the upper end of this restricted range it may be necessary to continue the infusion for more than one hour before the recovery occurs. If large enough doses are given, sufficient to cause complete blockade, recovery of the twitch may not occur.

Our results with prolonged application of decamethonium *in vivo* in the cat and in the rat (Creese & Maclagan, 1970) are comparable to those obtained by many authors in isolated muscle from the frog (Gissen & Nastuk, 1966), cat (Maclagan, 1962), rat (Thesleff, 1955; Gibberd, 1966; Harris & Leach, 1970), guinea-pig (Jenden, 1955), rabbit (Jenden, Kamijo & Taylor, 1954) and man (Creese, Dillon, Marshall, Sabawalla, Schneider, Taylor & Zinn, 1957). *In vitro*, as *in vivo*, the recovery of the twitch during prolonged application of the drug is critically dependent on the dose (Jenden *et al.*, 1954; Gissen & Nastuk, 1966).

Potential measurements are not available for most experiments on isolated muscles but it is known that in frog (Gissen & Nastuk, 1966) and in rat muscle (Thesleff, 1955) the membrane potential returned towards control levels while the drug was still in contact with the muscle. It appears, therefore, that recovery of both depolarization and muscle contractions during prolonged application of low doses of depolarizing drugs is a widespread phenomenon both *in vivo* and in isolated muscle preparations. There was, however, no sign of the secondary blockade which has been found *in vitro* and termed Phase II block by Taylor and his colleagues (Jenden *et al.*, 1954; Jenden, 1955).

When the radioactivity of the cat muscles was measured by scintillation methods at various times during the infusion, the results showed (1) that the drug accumulated in the muscle, (2) that the uptake increased progressively with time, (3) that muscles which were less sensitive to the action of decamethonium (soleus muscle) showed a smaller uptake of the drug than the more sensitive fast-contracting muscles, and (4) as might be expected, a procedure such as denervation of the muscle, which increases its sensitivity to all depolarizing drugs, greatly increased the uptake of the drug into the muscle. Similar results in rat and guinea-pig muscles have been obtained by Creese, Taylor & Case (1971).

The results of the experiments with labelled decamethonium also showed that the drug accumulated in the muscle to a greater extent than could be accounted for by its presence in the extracellular space. With this method of expressing uptake as a clearance in ml/g, the uptake into the extracellular space would be of the order of 0.1–0.2 ml/g, but in our experiments uptakes much greater than this were found in normal muscle and after denervation the values increased further. By use of frozen section autoradiography, which prevents movement of the water soluble drug and which permits more precise localization, the drug was found within the fibres. The resolution of the method was 1 µm (Creese & Maclagan, 1970) and there is the possibility that some of the drug is attached to the tubule system within the fibre. Longitudinal sections showed grains all along the fibres, with a concentration in the region of the end-plate and for several hundred microns on either side. There is some indication that decamethonium is entering the cells via the receptor-activated sodium channels; Creese & England (1970) and Humphrey (1975) both found that the permeability of rat muscle to decamethonium was similar to that of sodium and much lower than that of the potassium ion. In addition, tubocurarine prevents both the entry of the drug into the cells and the pharmacological action on the receptors.

Our finding that the uptake of decamethonium into the muscle cells increases progressively with time was of particular interest as the pharmacological effects of the drug on contraction and on the membrane potential were only transient. It has been suggested by others that the transient blockade and potential changes in isolated muscle preparations are due to the development of receptor desensitization (Thesleff, 1955; Gissen & Nastuk, 1966). However, this suggestion is difficult to reconcile with the finding that the drug continues to enter the cells, presumably via the sodium channels, even when the blockade has disappeared.

There are several possible ways to explain the waning pharmacological effect without postulating receptor desensitization. Stimulation of outward sodium pumping from the cell could explain the repolarization of the membrane while the sodium channels remain open. The stimulus to the sodium pump might be either an increase in the intracellular concentration of sodium, caused by decamethonium, or the resulting increase in potassium ion concentration in unstirred layers just outside the cell. Both of these mechanisms are known to be effective activators of the sodium pump in other tissues (Glynn, 1957, 1962; Glynn & Karlish, 1975).

Some evidence to support the presence of sodium pumping during the recovery phase comes from the work of Head (1975). He showed that in isolated cat muscles, ouabain, applied during the recovery phase after decamethonium-induced depolarization, slowed or prevented the normal recovery of the membrane potential. This effect may be species dependent as, in the frog under similar conditions, strophanthidin was ineffective (Terrar, 1974).

This problem cannot be resolved with the available evidence but the results obtained in the present experiments showing progressive uptake of decamethonium into cat muscle fibres do not appear to support the theory that receptor desensitization occurs during prolonged application of blocking doses of depolarizing drugs. At much higher concentrations, however, such as those produced during iontophoretic application, receptor desensitization may be an important factor (Thesleff, 1955; Gissen & Nastuk, 1966).

This research was supported by grants from the Medical Research Council and from the Wellcome Trust.

**References**

BOYD, I.A. & MARTIN, A.R. (1956). The end-plate potential in mammalian muscle. *J. Physiol., Lond.*, **132**, 74–91.

BURNS, B.D. & PATON, W.D.M. (1951). Depolarization of the motor end-plate by decamethonium and acetylcholine. *J. Physiol., Lond.*, **115**, 41–73.

CREESE, R., DILLON, J.B., MARSHALL, J., SABAWALLA, P.B., SCHNEIDER, D.J., TAYLOR, D.B. & ZINN, D.E. (1957). The effect of neuromuscular blocking agents on isolated human striated muscle. *J. Pharmac. exp. Ther.*, **119**, 485–494.

CREESE, R. & ENGLAND, J.M. (1970). Decamethonium in depolarized muscle and the effect of tubocurarine. *J. Physiol., Lond.*, **210**, 345–361.

CREESE, R. & MACLAGAN, J. (1967). Autoradiography of decamethonium in rat muscle. *Nature, Lond.*, **215**, 988–989.

CREESE, R. & MACLAGAN, J. (1970). Entry of decamethonium in rat muscle studied by autoradiography. *J. Physiol., Lond.*, **210**, 363–386.

CREESE, R. & TAYLOR, D.B. (1967). Entry of labelled carbachol in brain slices of the rat and the action of d-tubocurarine and strychnine. *J. Pharmac. exp. Ther.*, **157**, 406–419.

CREESE, R., TAYLOR, D.B. & CASE, R. (1970). Labeled decamethonium in denervated skeletal muscle. *J. Pharmac. exp. Ther.*, **176**, 418–422.

CREESE, R., TAYLOR, D.B. & TILTON, B. (1963). The influence of curare on the uptake of a neuromuscular blocking agent labelled with radioactive iodine. *J. Pharmac. exp. Ther.*, **139**, 8–17.

GIBBERD, F. (1966). Action of decamethonium in rat diaphragm. *Br. J. Pharmac.*, **28**, 128–136.

GISSEN, A.J. & NASTUK, W.L. (1966). The mechanisms underlying neuromuscular block following prolonged exposure to depolarizing agents. *Ann. N.Y. Acad. Sci.*, **135**, 184–194.

GLYNN, I.M. (1957). The action of cardiac glycosides on sodium and potassium movements in human red cells. *J. Physiol. (Lond.)*, **136**, 148–173.

GLYNN, I.M. (1962). Activation of adenosine-triphosphatase activity in a cell membrane by external potassium and internal sodium. *J. Physiol., Lond.*, **160**, 18–19P.

GLYNN, I.M. & KARLISH, S.J.D. (1975). The sodium pump. *Ann. Rev. Physiol.*, **37**, 13–55.

HARRIS, J. & LEACH, G. (1970). The effect of some foreign anions on suxamethonium blockade of the isolated rat diaphragm preparation. *Br. J. Pharmac.*, **38**, 517–529.

HEAD, SD. (1975). Depolarizing neuromuscular blocking drugs; an electrophysiological investigation in mammalian skeletal muscle. *Ph.D. Thesis, University of London.*

HUMPHREY, P.P.A. (1975). Decamethonium in the perfused and immersed rat diaphragm. *Br. J. Pharmac.*, **54**, 367–374.

JENDEN, D.J. (1955). The effect of drugs upon neuromuscular transmission in the isolated guinea pig diaphragm. *J. Pharmac. exp. Ther.*, **114**, 398–408.

JENDEN, D.J., KAMIJO, K. & TAYLOR, D.B. (1954). The action of decamethonium on the isolated rabbit lumbrical muscle. *J. Pharmac. exp. Ther.*, **111**, 229–240.

JEWELL, P.A. & ZAIMIS, E.J. (1954). A differentiation between red and white muscle in the cat based on the responses to neuromuscular blocking agents. *J. Physiol., Lond.*, **124**, 413–428.

MACLAGAN, J. (1962). A comparison of the responses of the tennuissimus muscle to neuromuscular blocking drugs in vivo and in vitro. *Br. J. Pharmac.*, **18**, 204–216.

MACLAGAN, J. (1976). Competitive neuromuscular blocking drugs. In *Neuromuscular Junction. Hefters Handbuch.* ed. Zaimis, E. Heidelberg: Springer-Verlag (in press).

POULSEN, H. & HOUGS, W. (1958). The effect of some curarizing drugs in unanaesthetized man. II. *Acta anaesth. scand.*, **2**, 107–115.

TERRAR, D.A. (1974). Influence of SKF525A congeners, strophanthidin and tissue culture media on desensitization in frog skeletal muscle. *Br. J. Pharmac.*, **51**, 529–568.

THESLEFF, S. (1955). The effects of acetylcholine, decamethonium and succinylcholine on neuromuscular transmission in the rat. *Acta physiol. scand.*, **34**, 386–392. 386–392.

VRBOVÁ, G. & MACLAGAN, J. (1966). A study of the increased sensitivity of denervated and re-innervated muscle to depolarizing drugs. *J. Physiol., Lond.*, **182**, 131–143.

WASER, P.G. (1966). Autoradiographic investigations of cholinergic and other receptors in the motor end-plate. In *Advances in Drug Research.* ed. Harper, N.J. & Simmonds, A.B. Vol. 3, pp. 81–120. London: Academic Press.

*(Received March 10, 1976)*

*Br. J. Pharmac.* (1976), **58**, 149–156

# THE EFFECT OF METIAMIDE ON ACID SECRETION STIMULATED BY GASTRIN, ACETYLCHOLINE AND DIBUTYRYL CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE IN THE ISOLATED WHOLE STOMACH OF THE RAT

K.T. BUNCE, M.E. PARSONS & NITA A. ROLLINGS
The Research Institute, Smith Kline & French Laboratories Limited,
Welwyn Garden City, Hertfordshire

1 An isolated stomach preparation from immature rats is described. The lumen of the stomach was perfused and the hydrogen ion activity of the perfusate recorded continuously.

2 The preparation gave dose-dependent responses to gastrin, acetylcholine and dibutyryl cyclic adenosine 3′,5′-monophosphate and these responses were readily reversed on washing out the agonist.

3 The acid secretory response to gastrin was inhibited by metiamide at concentrations of $10^{-5}$ M and $3 \times 10^{-5}$ M.

4 The acid secretory responses to acetylcholine and dibutyryl cyclic adenosine 3′,5′-monophosphate were not inhibited by concentrations of metiamide up to $10^{-3}$ M.

5 These findings are discussed in relation to the role of histamine in the control of gastric acid secretion.

## Introduction

The interrelationship of the three physiological gastric secretagogues, histamine, gastrin and acetylcholine in the stimulation of gastric secretion remains unresolved. Several lines of evidence implicate histamine as a mediator of gastrin-stimulated acid secretion. In the rat, the activity of gastric mucosal histidine decarboxylase (EC. 4.1.1.22) has been shown to be closely correlated with the serum gastrin level (Håkanson, Kroesen, Liedberg, Oscarson, Rehfeld & Stadil, 1974), and administration of gastrin diminishes the gastric mucosal histamine content (Haverback, Tecimer, Dyce, Cohen, Stubrin & Santa Ana, 1964; Kahlson, Rosengren, Svahn & Thunberg, 1964). Furthermore the histamine $H_2$-antagonists, metiamide and cimetidine, are potent inhibitors of pentagastrin-stimulated gastric acid secretion in several species (Wyllie & Hesselbo, 1973; Brimblecombe, Duncan, Durant, Emmett, Ganellin & Parsons, 1975; Burland, Duncan, Haggie, Hesselbo, Mills, Sharpe & Wyllie, 1975). The role of endogenous histamine in cholinergically-stimulated gastric acid secretion is less clear. Although the $H_2$-antagonists do inhibit cholinergically-stimulated secretion (Brimblecombe *et al.* 1975; Parsons, 1975), higher dose levels of the antagonists are required than for the inhibition of histamine- and pentagastrin-stimulated secretion and the interpretation of these studies is complicated by the release of endogenous gastrin. Also, experiments in the rat have led to disagreement about the direct cholinergic effect of vagal excitation on the mobilization of gastric mucosal histamine (Rosengren & Svensson, 1969; Håkanson & Liedberg, 1972). In recent years the role of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in the control of gastric function has stimulated considerable interest, and the subject has been extensively reviewed (Scratcherd & Case, 1973; Kimberg, 1974).

In the present study the isolated perfused stomach of the rat has been used for a quantitative investigation of the inhibitory effect of metiamide against gastrin-, acetylcholine- and dibutyryl cyclic adenosine 3′,5′-monophosphate- (db cyclic AMP) stimulated acid secretion in the absence of neural and indirect hormonal influences.

## Methods

Fed immature rats of either sex weighing between 35–45 g were anaesthetized with pentobarbitone (3 mg i.p.). The abdomen was opened and the oesophagus ligated close to the stomach. An incision was made in the rumen of the stomach and the contents washed out with warm Krebs-Henseleit solution. A second incision was then made at the pyloric sphincter and polythene cannulae were inserted and tied into the stomach via these incisions. The stomach was rapidly dissected out and placed immediately in a 10 ml organ bath containing Krebs-Henseleit solution at 37°C. The lumen of the the stomach was perfused by use of an H.R. Flow-Inducer (Watson-Marlow Ltd.), at a rate of 1 ml/min with a modified Krebs-Henseleit solution at 37°C. Both salt solutions were gassed with 95% $O_2$ and 5% $CO_2$. The effluent perfusate from the stomach was passed over a micro dual electrode (Russell pH Ltd.) clamped 20 cm higher than the stomach. Changes in pH were converted to a function of hydrogen ion activity by an antilog function generator (Research Institute Workshop, SK&F Laboratories Ltd.) and continuously recorded on a potentiometric pen recorder (Servoscribe). The rate of acid secretion was then expressed as $[H^+]$ mol $\times 10^{-8}$ per minute. The Krebs-Henseleit solution in the organ bath contained (mM) 119.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.03 $KH_2PO_4$ and 5.6 glucose. The modified lumen perfusate had a similar composition but with the $NaHCO_3$ and $KH_2PO_4$ omitted. All drugs were added in a volume not exceeding 0.5 ml, to the Krebs-Henseleit solution bathing the serosal surface of the stomach. After setting up the stomach preparation the basal $H^+$ output was allowed to stabilize, under control conditions and in the presence of metiamide, before the secretary responses to an agonist were investigated. The response to a single dose of an agonist was calculated as the amount of acid secreted at peak response minus the preceding basal level. Fresh solutions of pentagastrin, acetylcholine and metiamide were made each day in Krebs-Henseleit solution. A stock solution of db cyclic AMP at $10^{-2}$ M was prepared in Krebs-Henseleit solution and a stock solution of gastrin at 200 µg/ml was prepared in 0.05M $NH_4HCO_3$. Both stock solutions were stored at 4°C and diluted with Krebs-Henseleit solution as required.

### *Drugs*

The following drugs were used: gastrin (synthetic human gastrin I) and pentagastrin (Peptavlon, ICI Ltd.), acetylcholine chloride (BDH Ltd.), dibutyryl cyclic adenosine 3',5'-monophosphate (db cyclic AMP, Sigma Chemical Co. Ltd.), pentobarbitone (Nembutal, Abbott Laboratories Ltd.), metiamide (SK&F 92058) was synthesized in our own laboratories.

### *Analysis of results*

Results are expressed as mean ± s.e. mean. An analysis of variance was used to test the effect of metiamide on acid secretion stimulated by each

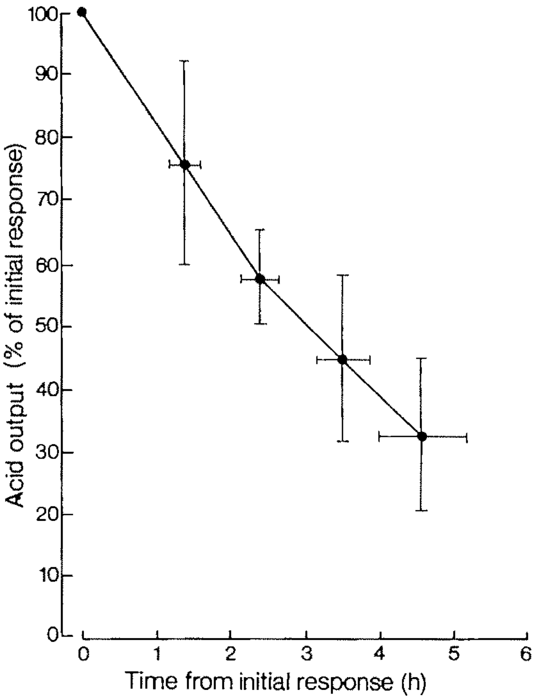


**Figure 1** Tachyphylaxis of the acid secretory response to repeated doses of pentagastrin at $10^{-7}$ M. The first response of each stomach was expressed as 100%, and the subsequent responses were recorded as a percentage of this first response. The figure records the mean times at which the doses of pentagastrin were administered. Each point is the mean of 4 to 5 observations. The vertical and horizontal bars show s.e. mean.

agonist. A *P*-value of less than 0.05 was considered to be significant.

## Results

For gastrin and acetylcholine, sequential dose-response curves have been constructed in order to determine the dose-range over which a linear dose-response relationship was obtained. Two suitable concentrations of each agonist were then chosen for carrying out the 2+2 assays, which were used to investigate the effect of metiamide on acid secretion. A prerequisite for this study was to establish that the preparation gave reproducible responses to a particular agonist. Therefore two control 2-point dose-response curves were constructed on eight stomach preparations for each agonist. These preliminary experiments were then used to determine whether

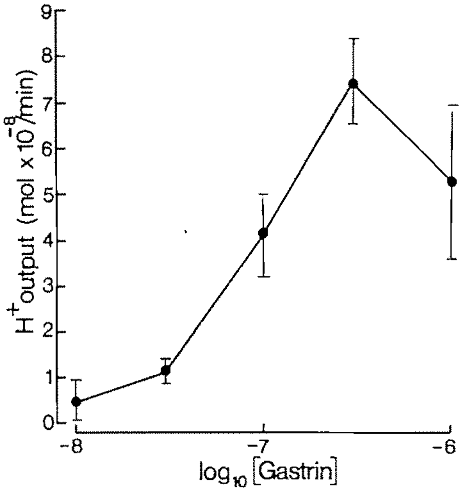


**Figure 2** Sequential dose-response curve to gastrin. Each point is the mean of 4 observations. Vertical lines show s.e. mean.

there were any time-related changes in the sensitivity of the preparation to an agonist, which might be unrelated to the presence of metiamide.

*Pentagastrin*

Preliminary experiments showed that the rat isolated stomach did not give reproducible responses to repeated doses of pentagastrin. The acid secretory responses to repeated doses of pentagastrin at $10^{-7}$ M are shown in Figure 1. Over a period of 5 h, during which five doses of pentagastrin were administered, the acid secretory response diminished by approximately 70%. The tachyphylaxis to pentagastrin made this secretagogue unsuitable for the construction of dose-response curves, and therefore gastrin was next investigated.

*Gastrin*

*Sequential dose-response curve.* The sequential dose-response curve for gastrin is shown in Figure 2. The threshold concentration was $10^{-8}$ M and the plot was linear over the range $3 \times 10^{-8}$ M to $3 \times 10^{-7}$ M. A concentration of gastrin of $10^{-6}$ M gave a submaximal response. Doses of $10^{-7}$ M and $3 \times 10^{-7}$ M were therefore used for the construction of 2-point dose-response curves, the doses being administered in a randomized order.

*Reproducibility of response.* Although the acid secretory response of the isolated stomach also showed some tachyphylaxis to gastrin, particularly between the first two responses to the hormone, this was less marked than with pentagastrin. For this reason the first dose of gastrin was considered to be a 'priming' dose, and if a dose of $10^{-7}$ M gastrin was given for this purpose the subsequent four responses were then sufficiently reproducible for the construction of the 2-point dose-response curves.

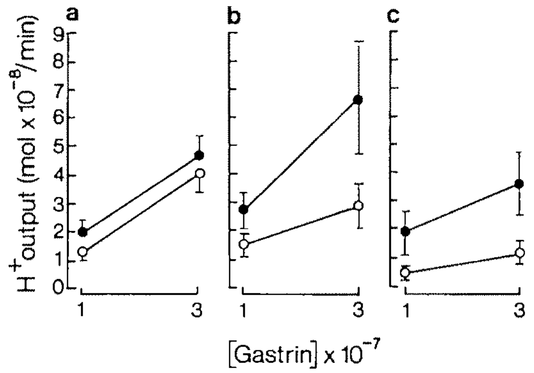


**Figure 3** Dose-response curves to gastrin in the presence and absence of metiamide. Vertical lines show s.e. mean. Analyses of variance on these data provided the following information. (a) Control ($n=8$); the second·curve (○) was not significantly displaced from the first curve (●); the slopes of the two lines were not significantly different; (b) $10^{-5}$ M metiamide ($n=9$); (c) $3 \times 10^{-5}$ M metiamide ($n=7$). In each case the slope of the line in the presence of metiamide (○) was not significantly different from that obtained with gastrin alone (●). The dose-response curves to gastrin in the presence of metiamide ($10^{-5}$ M and $3 \times 10^{5}$ M) were significantly displaced from their respective control curves.

*The effect of metiamide.* A study of the effect of $H_2$-receptor blockade on the response to gastrin was made with two concentrations of metiamide ($10^{-5}$ M and $3 \times 10^{-5}$ M). For this purpose two 2-point gastrin dose-response curves were constructed on each stomach preparation. A control dose-response curve was established and each stomach was then equilibrated in Krebs-Henseleit solution containing the appropriate concentration of metiamide for approximately 1 h, and then the second curve constructed. The mean dose-response curves obtained in these experiments are shown in Figure 3. The control data (Figure 3a) show that there were no significant time-related changes in the sensitivity of the preparation to gastrin, and this result indicates that the omission of the first 'priming' gastrin dose from the assay was expedient. The results presented in Figure 3 show that metiamide is an effective inhibitor of gastrin-stimulated acid secretion in this preparation, although the pattern of inhibition differs from that obtained when histamine was used as the agonist (Bunce &

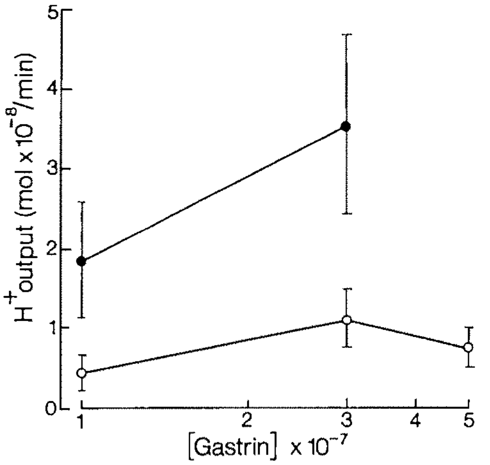


**Figure 4** The acid secretory response to $5 \times 10^{-7}$ M gastrin in the presence of metiamide ($3 \times 10^{-5}$ M). Control (●), metiamide (O). Each point is the mean of 7 observations. Vertical lines show s.e. mean.

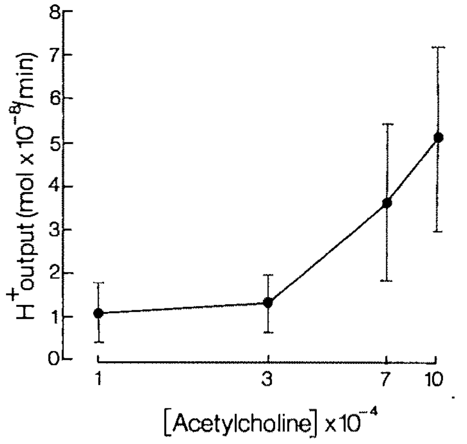


**Figure 5** Sequential dose-response curve to acetylcholine. Each point is the mean of 4 observations. Vertical lines show s.e. mean.

Parsons, 1976). The latter authors showed that a parallel displacement of the histamine dose-response curve to the right was obtained which enabled dose-ratios to be calculated; in the present study the concentration of gastrin was increased to $5 \times 10^{-7}$M in the presence of metiamide in an effort to provide comparable data. However, as can be seen in Figure 4, the response to this latter concentration of gastrin was less than that obtained to a concentration of $3 \times 10^{-7}$M, and the resultant depression of the maximum response compared to the control suggests a non-competitive type of antagonism. In the control dose-response curves in the present experiments, concentrations of gastrin greater than $3 \times 10^{-7}$M were not used to determine whether a similar reduction in response was obtained. However, extrapolation from the sequential dose-response curve (Figure 2) does indicate that under control conditions also, a concentration of gastrin above $3 \times 10^{-7}$M gives a submaximal response. Calculation of dose-ratios for comparison of the present results with those obtained with histamine was therefore impossible.

### *Acetylcholine*

*Sequential dose-response curve.* The sequential dose-response curve for acetylcholine is shown in Figure 5. The threshold concentration of acetylcholine was $10^{-4}$ M, and the plot was linear over the range $3 \times 10^{-4}$ M to $10^{-3}$ M. A concentration of acetylcholine of $3 \times 10^{-3}$ M not only failed to stimulate acid secretion, but also diminished the basal rate of acid output. Doses of $3 \times 10^{-4}$ M and $7 \times 10^{-4}$ M were therefore used for the construction of 2-point dose-response curves, and these doses were administered in a randomized order.

*Reproducibility of response.* As with gastrin, the first dose of acetylcholine produced a secretory response which was greater than the subsequent responses to similar doses of this compound. Therefore a 'priming' dose of $3 \times 10^{-4}$ M acetylcholine was routinely used in these experiments. However, unlike gastrin, the responses subsequent to that produced by the 'priming' acetylcholine dose showed a slight tendency to increase during the course of an experiment.

*The effect of metiamide.* Two 2-point dose-response curves to acetylcholine were constructed on each stomach preparation. The experimental procedure was as described for gastrin. Metiamide was used at concentrations of $3 \times 10^{-5}$ M, $10^{-4}$ M and $10^{-3}$ M. The mean dose-response curves obtained in these experiments are recorded in Figure 6. The control curves obtained in the absence of metiamide (Figure 6a) show that there was a small but insignificant shift of the dose-response curve to the left. Similar changes were also seen in the presence of metiamide at concentrations of $3 \times 10^{-5}$ M and $10^{-4}$ M (Figure 6b and c), and a further increase of the metiamide concentration to $10^{-3}$ M produced comparable results. At each concentration investigated, metiamide failed to produce a shift of the dose-response curve to the right, and this result indicates that metiamide is not an inhibitor of

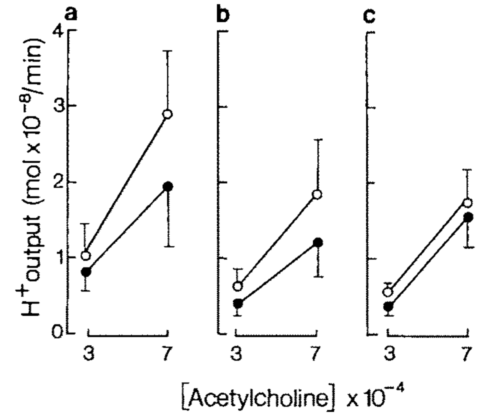


**Figure 6** Dose-response curves to acetylcholine in the presence and absence of metiamide. Vertical lines show s.e. mean. Analyses of variance on these data provided the following information. (a) Control ($n=8$); the second curve (O) was not significantly displaced from the first curve (●); the slopes of the two lines were not significantly different; (b) $3\times10^{-5}$ M metiamide ($n=7$); (c) $10^{-4}$ M metiamide ($n=8$). In each case the slope of the line in the presence of metiamide (O) was not significantly different from that obtained with acetylcholine alone (●). The dose-response curves to acetylcholine in the presence of metiamide ($3\times10^{-5}$ M and $10^{-4}$ M) were not significantly displaced from the respective control curves.

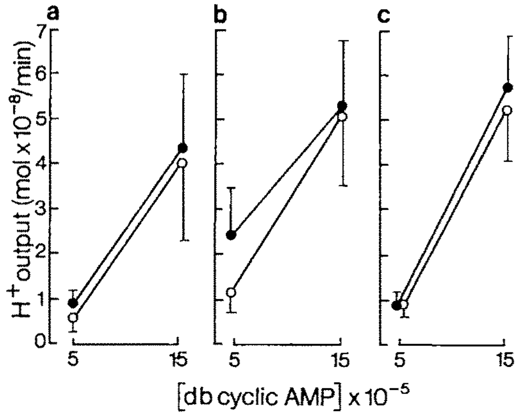


**Figure 7** Dose-response curves to dibutyryl cyclic adenosine 3′,5′-monophosphate (db cyclic AMP) in the presence and absence of metiamide. Vertical lines show s.e. mean. Analyses of variance on these data provided the following information. (a) Control ($n=8$); the second curve (O) was not significantly displaced from the first curve (●); the slopes of the two lines were not significantly different; (b) $10^{-4}$ M metiamide ($n=9$); (c) $10^{-3}$ M metiamide ($n=7$). In each case the slope of the line in the presence of metiamide (O) was not significantly different from that obtained with db cyclic AMP alone (●). The dose-response curves to db cyclic AMP in the presence of metiamide ($10^{-4}$ M and $10^{-3}$M) were not significantly displaced from the respective control curves.

acetylcholine-stimulated secretion in the isolated stomach preparation.

### *Dibutyryl cyclic adenosine 3′,5′-monophosphate*

Preliminary experiments with db cyclic AMP showed that the threshold concentration was $5\times10^{-6}$ M, and that concentrations of $5\times10^{-5}$ M and $1.5\times10^{-4}$ M gave dose-related responses which could be used to investigate the effect of metiamide.

*The effect of metiamide.* The experimental procedure was as described for gastrin and acetylcholine. Metiamide was used at concentrations of $10^{-4}$ M and $10^{-3}$ M, and the mean dose-response curves obtained in these experiments are shown in Figure 7. Control dose-response curves in the absence of metiamide (Figure 7a) showed that there were no significant time-related changes in the sensitivity of the preparation to db cyclic AMP. Metiamide, at the high concentrations used, did not inhibit db cyclic AMP-stimulated acid secretion in this preparation.

## Discussion

The present experiments on the rat isolated stomach show that the preparation is a useful tool for the elucidation of the control of gastric function. One problem associated with the preparation which was immediately apparent is the tachyphylaxis observed to repeated doses of pentagastrin. This phenomenon of tachyphylaxis to gastric secretagogues has also been recorded by other workers both *in vivo* and *in vitro*. Kasbekar (1972) found that the amphibian isolated gastric mucosa exhibited tachyphylaxis to repeated doses of pentagastrin. In the anaesthetized cat repeated injections of a gastrin extract also produced tachyphylaxis (Uvnäs, 1945; Emas, 1960; Blair, Harber, Lake & Reed, 1963), although in the anaesthetized lumen-perfused rat a progressive increase in the sensitivity of the preparation to gastrin has been demonstrated (Lai, 1964; Parsons, 1969). This latter observation is in direct contrast with the results obtained on the isolated stomach of the rat. In the present work, the tachyphylaxis to pentagastrin was more severe than that to gastrin; an observation which cannot be explained at the present time.

Some workers have reported the failure of pentagastrin to stimulate acid secretion from mammalian isolated gastric mucosa preparations, e.g. piglet gastric mucosa (Forte, Forte & Machen, 1975) and guinea-pig gastric mucosa (Spencer, 1974). However, acid secretion has previously been obtained in response to pentagastrin from *in vitro* rat gastric mucosa and stomach preparations. Brennan, Arbakov, Stefankiewicz & Groves (1975) obtained a maximum secretory rate of approximately 33 μEq/h above basal in response to $3 \times 10^{-5}$ M pentagastrin and Wan, Assem & Schild (1974) recorded a maximum rate of approximately 12 μEq $cm^{-2}$ 30 $min^{-1}$ above basal to $1.3 \times 10^{-6}$ M pentagastrin. In this investigation the isolated stomach of the rat gave a maximum acid secretory response of $7.5 \times 10^{-8}$ mol $min^{-1}$ (equivalent to 4.5 μEq/h) in response to $3 \times 10^{-7}$ M gastrin (Figure 2), and for comparison the surface area of the acid secreting mucosa in the immature rat was approximately 2 $cm^2$. However, the preparations of Wan *et al.* (1974) and Brennan *et al.* (1975) also gave higher acid secretory responses to histamine than the preparation described by Bunce & Parsons (1976). Possible explanations for these differences are that in this study immature animals were used, whereas both Wan *et al.* (1974) and Brennan *et al.* (1975) used mature rats. The techniques employed also differed considerably. The latter groups of workers both used static systems, whereas a perfusion technique was used in the present work. Also, of course, it may not be valid to compare the effects of pentagastrin and gastrin.

The reduction of the maximal response to gastrin in the presence of metiamide (Figure 4) is compatible with a non-competitive type of inhibition, and is similar to results obtained *in vivo* using pentagastrin in the Heidenhain pouch rat (Lundell, 1975), and by Black (1973) using the Heidenhain pouch dog. However metiamide has been shown to act like a competitive antagonist at $H_2$-receptors (Parsons, 1973; Bunce & Parsons, 1976), and this situation led Black (1973) to postulate that canine gastric mucosa possesses two types of gastrin receptor. One type of receptor, whose effects are mediated by $H_2$-receptors, is excitatory, and the other, which is not affected by metiamide, is inhibitory. Under these circumstances it is possible that metiamide, a competitive $H_2$-receptor antagonist, would depress the maximum secretory response to gastrin as seen in the present study.

There are few reports on the inhibition of gastrin- or pentagastrin-stimulated acid secretion by an $H_2$-antagonist *in vitro*. Wan *et al.* (1974) reported that metiamide inhibited the acid secretory response to pentagastrin in the isolated stomach of the rat and Schofield, Tepperman & Tepperman (1975) found that metiamide ($5 \times 10^{-3}$ M) inhibited pentagastrin-stimulated acid secretion in the *in vitro* kitten fundic mucosa. Both of these groups of workers investigated the effect of metiamide on one dose level only of pentagastrin, and the test and control experiments were carried out on separate mucosa preparations. In this investigation two dose levels of gastrin were used, and both test and control dose-response curves were constructed on each stomach. However, the results obtained in the present work do confirm the observations of Wan *et al.* (1974) and Schofield *et al.* (1975), and provide additional evidence to that reported *in vivo* (Parsons, 1975) that the acid secretory response to gastrin is probably mediated at least in part through a histaminergic pathway.

There is little information available about acid secretion stimulated by db cyclic AMP and cholinomimetic compounds in mammalian isolated stomach and gastric mucosa preparations. In the present experiments both acetylcholine ($10^{-3}$ M) and db cyclic AMP ($1.5 \times 10^{-4}$ M) stimulated an acid secretory response of approximately 5.0 mol × $10^{-8}$/min (equivalent to 3.0 μEq/h). A similar rate of acid secretion (2.63 μEq $cm^{-2}$ $h^{-1}$) was obtained by Schofield *et al.* (1975) from the isolated kitten fundic mucosa in response to acetylcholine ($10^{-4}$ M). Higher rates of acid secretion were obtained by Brennan *et al.* (1975) on the isolated rat stomach in response to both bethanechol ($2.2 \times 10^{-4}$ M) and db cyclic AMP ($10^{-4}$ M), and also by Fromm, Schwartz & Quijano (1975) on the rabbit isolated gastric mucosa in response to db cyclic AMP ($1.2 \times 10^{-3}$ M). However, it is of interest that both the latter groups of workers used stomachs from mature animals, whereas Schofield *et al.* (1975) used stomachs from immature animals as in the present investigation.

*In vivo*, cholinergic excitation causes acid secretion by a direct action on the fundic mucosa, and also via the release of gastrin from the pyloric antrum (Csendes, Walsh & Grossman, 1972). *In vitro*, there is no information about the release of gastrin from the rat stomach although it is unlikely that sufficient amounts of gastrin would be released into the serosal solution for biological action, and on this basis the isolated stomach of the rat enables a study of the direct secretagogue effect of acetylcholine. The failure of metiamide to inhibit acetylcholine-stimulated acid secretion *in vitro* (Figure 6) has also been shown by Schofield *et al.* (1975) and agrees well with previous observations on guinea-pig ileum where metiamide has no specific interaction with muscarinic receptors (Black & Spencer, 1973), although the result is in contrast with the view held by Kowalewski & Kolodej (1974) that metiamide does exhibit some cholinoceptor blocking activity. Also, the ineffectiveness of metiamide against acetylcholine-stimulated acid secretion indicates that acetylcholine *per se* does not mobilize gastric mucosal histamine, but stimulates acid secretion through a direct cholinergic pathway.

Interaction of histamine with $H_2$-receptors influences the level of cyclic AMP in rat gastric mucosa. Exogenous histamine increases the activity of gastric mucosal adenylate cyclase and elevates the level of cyclic AMP in rat gastric mucosa, and these changes can be prevented by $H_2$-antagonists (McNeill & Verma, 1974; Ruoff & Sewing, 1975). The latter experiments show that cyclic AMP operates at a position 'distal' to histamine in the chain of events which lead to the secretion of acid. This conclusion is supported by the results obtained in the present study in which metiamide failed to inhibit db cyclic AMP-stimulated acid secretion, and similar results have been obtained by other workers using isolated mammalian stomach and gastric mucosa preparations (Fromm *et al.* 1975; Hearn & Main, 1975; Wan, 1976).

We are indebted to Mr J.V. Smart for help with the statistical calculations.

## References

BLACK, J.W. (1973). Speculation about the nature of the antagonism between metiamide and pentagastrin. In *International Symposium on histamine $H_2$-receptor antagonists,* ed. Woods, C.J. & Simkins, M.A. pp. 219–224. Welwyn Garden City: Research and Development Division, Smith Kline & French Laboratories Ltd.

BLACK, J.W. & SPENCER, K.E.V. (1973). Metiamide in systematic screening tests. In *International Symposium on histamine $H_2$-receptor antagonists* ed. Woods, C.J. & Simkins, M.A., pp. 23–27. Welwyn Garden City: Research and Development Division, Smith Kline & French Laboratories Ltd.

BLAIR, E.L., HARPER, A.A., LAKE, H.J. & REED, J.D. (1963). Characteristics of responses to 'gastrin' extracts. *J. Physiol., Lond.,* **165**, 81–82P.

BRENNAN, F.T., ARBAKOV, D., STEFANKIEWICZ, J.S. & GROVES, W.G. (1975). Acid secretory effects of pentagastrin, histamine, urecholine, DBcAMP and cGMP in isolated stomachs of fed and fasted rats. *Proc. Soc. exp. Biol. Med.,* **149**, 725–730.

BRIMBLECOMBE, R.W., DUNCAN, W.A.M., DURANT, G.J., EMMETT, J.C., GANELLIN, C.R. & PARSONS, M.E. (1975). Cimetidine—A non-thiourea $H_2$-receptor antagonist. *J. Int. med. Res.,* **3**, 86–92.

BUNCE, K.T. & PARSONS, M.E. (1976). A quantitative study of metiamide, a histamine $H_2$-antagonist, on the isolated whole rat stomach. *J. Physiol., Lond.* (in press).

BURLAND, W.L., DUNCAN, W.A.M., HAGGIE, S.J., HESSELBO, T., MILLS, J.G., SHARPE, P.C. & WYLLIE, J.H. (1975). Pharmacological evaluation of cimetidine, a new histamine $H_2$-receptor antagonist, in healthy man. *Br. J. clin. Pharmac.,* **2**, 481–486.

CSENDES, A., WALSH, J.H. & GROSSMAN, M.I. (1972). Effects of atropine and of antral acidification on gastrin release and acid secretion in response to insulin and feeding in dogs. *Gastroenterology,* **63**, 257–263.

EMAS, S. (1960). Gastric secretory responses to repeated intravenous infusions of histamine and gastrin in non-anaesthetised and anaesthetised gastric fistula cats. *Gastroenterology,* **39**, 771–782.

FORTE, J.G., FORTE, G.M. & MACHEN, T.E. (1975). Histamine-stimulated hydrogen ion secretion by in vitro piglet gastric mucosa. *J. Physiol., Lond.,* **244**, 15–31.

FROMM, D., SCHWARTZ, J.H. & QUIJANO, R. (1975). Effects of cyclic adenosine 3′,5′-monophosphate and related agents on acid secretion by isolated rabbit gastric mucosa. *Gastroenterology,* **69**, 453–462.

HÅKANSON, R., KROESEN, J.H., LIEDBERG, G., OSCARSON, J., REHFELD, J.F. & STADIL, F. (1974). Correlation between serum gastrin concentration and rat stomach histidine decarboxylase activity. *J. Physiol., Lond.,* **243**, 483–498.

HÅKANSON, R. & LIEDBERG, G. (1972). Effect of pentagastrin and feeding on gastric histidine decarboxylase activity in the antrectomised rat. *Eur. J. Pharmac.,* **18**, 31–36.

HAVERBACK, B.J., TECIMER, L.B., DYCE, B.J., COHN, M., SUBRIN, M.I. & SANTA ANA, A.D. (1964). The effect of gastrin on stomach histamine in the rat. *Life Sci.,* **3**, 637–649.

HEARN, R. & MAIN, I.H.M. (1975). Stimulation and inhibition of acid secretion from the rat isolated gastric mucosa. *J. Physiol., Lond.,* **251**, 11–12P.

KAHLSON, G., ROSENGREN, E., SVAHN, E. & THUNBERG, R. (1964). Mobilisation and formation of histamine in the gastric mucosa as related to acid secretion. *J. Physiol., Lond.,* **174**, 400–416.

KASBEKAR, D.K. (1972). Secretagogue-induced tachyphylaxis of gastric $H^+$ secretion and its reversal. *Am. J. Physiol.,* **223**, 294–299.

KIMBERG, D.V. (1974). Cyclic nucleotides and their role in gastrointestinal secretion. *Gastroenterology,* **67**, 1023–1064.

KOWALEWSKI, K. & KOLODEJ, A. (1974). Effect of metiamide, a histamine antagonist of $H_2$-receptors, on acid secretion of isolated canine stomach perfused with homologous blood. *Pharmacology,* **11**, 207–212.

LAI, K.S. (1964). Studies on gastrin. Part 1. A method of biological assay of gastrin. *Gut,* **5**, 327–333.

LUNDELL, L. (1975). Displacement by metiamide of the dose-response curves to pentagastrin and methacholine in the conscious rat. *Br. J. Pharmac.,* **54**, 507–509.

McNEILL, J.H. & VERMA, S.C. (1974). Stimulation of rat gastric adenylate cyclase by histamine and histamine analogues and blockade by burimamide. *Br. J. Pharmac.,* **52**, 104–106.

PARSONS, M.E. (1969). Quantitative studies of drug induced acid gastric secretion. Ph.D. Thesis, University of London.

PARSONS, M.E. (1973). The evidence that the inhibition of histamine-stimulated gastric secretion is a result of the blockade of histamine $H_2$-receptors. In *International symposium on histamine $H_2$-receptor antagonists*, ed. Woods, C.J. & Simkins, M.A. pp. 207–217. Welwyn Garden City: Research and Development Division, Smith Kline & French Laboratories Ltd.

PARSONS, M.E. (1975). Antagonists of the histamine $H_2$-receptors. In *Topics in Gastroenterology*. Vol. 3. ed. Truelove, S.C. & Goodman, M.J. Oxford: Blackwell Scientific Publications.

ROSENGREN, E. & SVENSSON, S.E. (1969). The role of the antrum and the vagus nerve in the formation of gastric mucosal histamine. *J. Physiol., Lond.*, **205**, 275–288.

RUOFF, H.-J. & SEWING, K.-Fr. (1975). Influence of atropine, metiamide and vagotomy on cAMP of resting and stimulated gastric mucosa. *Eur. J. Pharmac.*, **32**, 227–232.

SCHOFIELD, B., TEPPERMAN, B.L. & TEPPERMAN, F.S. (1975). Effect of metiamide on acid secretion of kitten fundic mucosa *in vitro*. *Gastroenterology*, **68**, A-125.

SCRATCHERD, T. & CASE, R.M. (1973). The role of adenyl cyclase in the gastrointestinal tract. In *International Encyclopedia of Pharmacology and Therapeutics*. Section 39A. Pharmacology of Gastrointestinal Motility and Secretion. Vol. 2. ed. Holton, P., pp. 547–612. Oxford: Pergamon Press.

SPENCER, J. (1974). Gastric secretion in the isolated stomach of the guinea pig. *J. Physiol., Lond.*, **237**, 1–3P.

UVNÄS, B. (1945). Further attempts to isolate a gastric secretory excitant from the pyloric mucosa of pigs. *Acta physiol. scand.*, **9**, 296–305.

WAN, B.Y.C. (1976). Effects of dibutyryl cyclic AMP and phosphodiesterase inhibitors on acid secretion by mouse stomach *in vitro*. *Br. J. Pharmac.*, **56**, 357–358P.

WAN, B.Y.C., ASSEM, E.S.K. & SCHILD, H.O. (1974). Inhibition of *in vitro* stimulated gastric acid secretion by a histamine $H_2$-receptor antagonist, metiamide. *Eur. J. Pharmac.*, **29**, 83–88.

WYLLIE, J.H. & HESSELBO, T. (1973). Inhibition of gastric secretion in man by metiamide. In *International Symposium on histamine $H_2$-receptor antagonists*, ed. Woods, C.J. & Simkins, M.A., pp. 371–382. Welwyn Garden City: Research and Development Division, Smith Kline & French Laboratories Ltd.

*(Received March 15, 1976.*
*Revised April 6, 1976)*

*Br. J. Pharmac.* (1976), **58**, 157–159

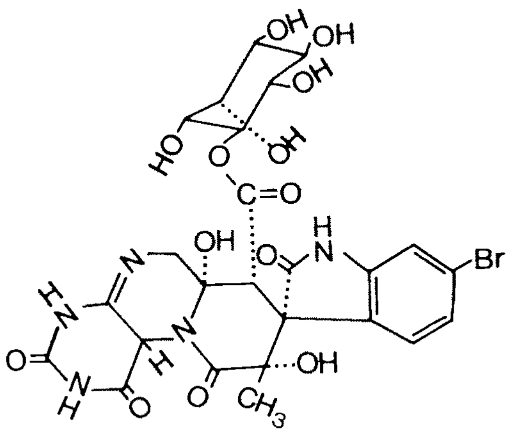
# ACTION OF SURUGATOXIN ON NICOTINIC RECEPTORS IN THE SUPERIOR CERVICAL GANGLION OF THE RAT

D.A. BROWN & J. GARTHWAITE
Department of Pharmacology, The School of Pharmacy, University of London, 29/39, Brunswick Square, London, WC1N 1AX

E. HAYASHI & S. YAMADA
Department of Pharmacology, Shizuoka College of Pharmaceutical Sciences, 2-2-1 Oshika, Shizuoka, Japan

Surugatoxin (SGTX, 0.1–2 μM) reversibly depressed orthodromic transmission and antagonized the depolarizing action of carbachol on the isolated superior cervical ganglion of the rat. The apparent dissociation equilibrium constant against carbachol-induced depolarization (measured in the presence of hyoscine) was 58 and 76 nM determined at 0.2 and 2 μM respectively. SGTX (2 μM) did not reduce the depolarizing effects of (±)-muscarine, γ-aminobutyric acid or angiotensin, but did reduce that to 5-hydroxytryptamine. Release of [$^3$H]-acetylcholine following repetitive (10 Hz) preganglionic sympathetic stimulation was maintained in the presence of 2 μM SGTX. It is concluded that SGTX has a high and selective affinity for ganglionic nicotinic receptors.

**Introduction** Surugatoxin (SGTX) is a toxin extracted from the Japanese ivory mollusc *Babylonia japonica*, with a molecular formula $C_{25}H_{26}N_5O_{13}Br.7H_2O$ (molecular weight 810.5) and molecular structure as follows (Kosuge, Zenda & Ochiai, 1972):



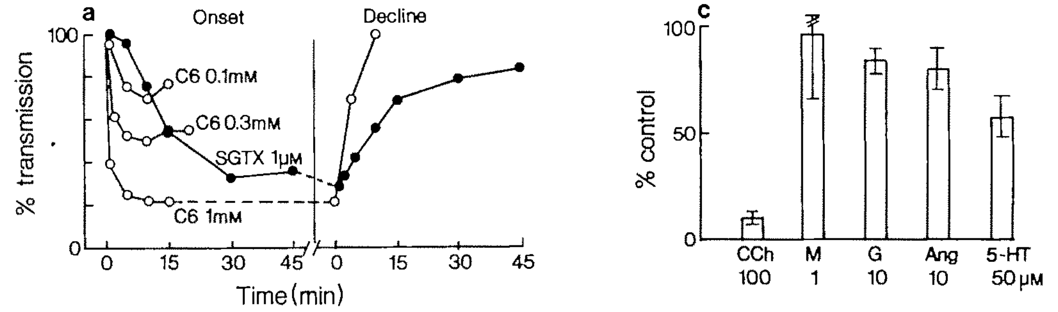
Hayashi & Yamada (1975) found SGTX to be a potent antagonist of nicotinic agonists in the cat superior cervical ganglion and guinea-pig ileum, with little effect on skeletal muscle or smooth muscle receptors, and concluded that poisoning resulted from ganglion-block.

We have recently investigated the action of SGTX on isolated ganglia of the rat and have confirmed its high affinity for nicotinic receptors therein.

**Methods** Superior cervical ganglia were isolated from rats anaesthetized with urethane and were maintained in Krebs solution at ambient temperature (27–30°C) bubbled with 95% $O_2$ and 5% $CO_2$ mixture (Brown, Brownstein & Scholfield, 1972). Depolarizing responses to agonists were recorded potentiometrically by the use of surface electrodes and a superfusion technique (Brown & Marsh, 1975). Ganglionic action potentials elicited by preganglionic nerve stimulation were recorded with a triple-chamber bath (Bowery & Tullett, 1975) and capacitor-coupled amplification (time-constant 10 seconds). In one experiment the triple-chamber bath was also used to measure the release of acetylcholine by preganglionic stimulation, as [$^3$H]-acetylcholine (see Brown, Halliwell, Jones & Quilliam, 1970). The ganglion chamber was perfused for 2 h with 0.8 μM [$^3$H]-choline and the subsequent release of tritium by 4 min periods of 10 Hz preganglionic stimulation measured. [$^3$H]-choline was obtained from the Radiochemical Centre, Amersham. Pure crystalline SGTX was a gift from Professor T. Kosuge.

**Results** SGTX (1 μM) produced a slowly-developing block of the response to orthodromic transmission (Figure 1a). Though slower in both onset and decline, its effect resembled that of hexamethonium in the following respects: (i) the N-wave (synaptic potential) was strongly depressed; and (ii) block was intensified on raising the stimulus frequency from 1 Hz to 10 Hz.

Dose-depolarization curves for carbachol (measured in the presence of 2.9 μM hyoscine) were



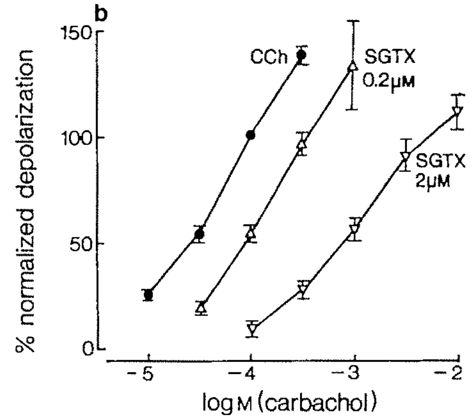


**Figure 1** (a) Effects of hexamethonium (C6) and surugatoxin (SGTX) on the amplitude of the ganglionic action potential in response to supramaximal 1 Hz, 1 ms preganglionic nerve stimulation. Ordinates, % of initial amplitude; abscissae, time after adding and washing out the blocking agent. (b) Depolarizations produced by 1 min applications of carbachol at 30 min intervals in the absence and presence of surugatoxin (SGTX). Ordinates: depolarization, expressed as % of that produced by 100 μM carbachol in the absence of SGTX (●). Abscissae: log molar carbachol concentration. Each point is the mean of 4 control experiments or of 3 experiments in SGTX solution (vertical lines show standard errors). (c) Depolarizing responses to standard concentrations of carbachol (100 μM, CCh), (±)-muscarine (M, 1 μM), γ-aminobutyric acid (G, 100 μM), angiotensin (Ang, 10 μM) and 5-hydroxytryptamine (5-HT, 50 μM) in the presence of 2 μM SGTX, expressed as % of control responses (means and standard errors of 4 experiments).

shifted in an approximately parallel manner by SGTX (Figure 1b). Apparent dissociation constants measured at equilibrium block for SGTX were 58 nM (measured from the shift produced by 0.2 μM SGTX) and 76 nM (measured at 2 μM). The apparent dissociation constant for hexamethonium against carbachol was 7.6 μM (at 10 μM hexamethonium) and 19.7 μM at 100 μM hexamethonium. SGTX did not depolarize the ganglion.

At a concentration of 2 μM, SGTX did not significantly reduce the depolarizations produced by γ-aminobutyric acid (GABA, 100 μM), (±)-muscarine (1 μM), or angiotensin (10 μM), but appeared to reduce the response to 5-hydroxytryptamine (50 μM) by some 44% (Figure 1c).

In one test, SGTX (2 μM) did not reduce the neurally-evoked release of tritium from a ganglion previously labelled with [$^3$H]-choline; in the same experiment, release was reversibly suppressed by a 20 mM[$Mg^{2+}$], $Ca^{2+}$-free solution.

**Discussion** The present observations confirm the high affinity of SGTX for ganglionic nicotinic receptors deduced by Hayashi & Yamada (1975) from tests on the guinea-pig ileum: its apparent affinity constant is at least two orders of magnitude greater than that of hexamethonium. The slow decline of ganglion block would accord with this. Slow onset is unlikely to be due to restricted diffusion *per se*, since SGTX is not a large molecule; rather, it may reflect a buffering effect of receptor-binding on the interstitial concentration (as with tetrodotoxin: cf. Colquhoun, Henderson & Ritchie, 1972).

There was some suggestion of antagonism to 5-hydroxytryptamine; though requiring further quantitation, this also accords with the observation of Hayashi & Yamada (1975) on the guinea-pig ileum. Other ganglionic receptors for muscarine, GABA and angiotensin appear resistant to SGTX. This, coupled with an inability to reduce acetylcholine release, suggests that SGTX is a fairly specific 'competitive' nicotinic ganglion-blocking agent.

SGTX is of appreciable interest as a 'neurotoxin' with a selective affinity for ganglionic nicotinic receptors. Its uniqueness in this respect is emphasized by the fact that the polypeptide snake venoms so effective at motor end-plates are generally quite inactive at ganglia (Magazanik, Ivanov & Lukomskaya, 1974; Brown, D.A., unpublished observations). The action of SGTX highlights the distinction between these two types of nicotinic receptor.

We are grateful to Professor T. Kosuge (Shizuoka College of Pharmaceutical Sciences, Shizuoka, Japan) for the gift of surugatoxin, to Dr N.G, Bowery for undertaking the acetylcholine-release experiment and to S. Marsh for his assistance. The investigation was aided by a grant from the American Medical Association Education and Research Foundation to D.A.B.

## References

BOWERY, N.G. & TULETT, D. (1975). A technique for the simultaneous measurement of effluent radioactivity and electrical responses in the rat superior cervical ganglion and its pre- and postganglionic trunks *in vitro*. *J. Physiol., Lond.*, **246**, 20–21P.

BROWN, D.A., BROWNSTEIN, M.J. & SCHOLFIELD, C.N. (1972). Origin of the after-hyperpolarization that follows removal of depolarizing agents from the isolated superior cervical ganglion of the rat. *Br. J. Pharmac.*, **44**, 651–671.

BROWN, D.A., HALLIWELL, J.V., JONES, K.B. & QUILLIAM, J.P. (1970). Evidence against a presynaptic action of acetylcholine during ganglionic transmission. *Nature New Biol., Lond.*, **266**, 958–959.

BROWN, D.A. & MARSH, S. (1975). A very simple method for recording ganglion depolarization. *J. Physiol., Lond.*, **246**, 24–26P.

COLQUHOUN, D., HENDERSON, R. & RITCHIE, J.M. (1972). The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol., Lond.*, **227**, 95–126.

HAYASHI, E. & YAMADA, S. (1975). Pharmacological studies on surugatoxin, the toxic principle from Japanese Ivory mollusc (*Babylonia japonica*). *Br. J. Pharmac.*, **53**, 207–215.

KOSUGE, T., ZENDA, H. & OCHIAI, A. (1972). Isolation and structure determination of a new marine toxin, surugatoxin, from the Japanese Ivory shell, *Babylonia japonica*. *Tetrahedron Letters*, **25**, 2545–2548.

MAGAZANIK, L.G., IVANOV, A.Ya. & LUKOMSKAYA, N.Ya. (1974). The effect of snake venom polypeptides on cholinoreceptors in isolated rabbit sympathetic ganglia. *Neurofiziol.*, **6**, 652–656. [In Russian.]

*(Received May 5, 1976.)*

# INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac*, **50**, 3-23, may also be obtained. Specific points to note are:

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors **in alphabetical order**. A short title of not more than 50 letters should also be suggested.

2. **Statement** Papers are accepted only if accompanied by a statement that they have not been and will not be published in whole or in part in any other Journal.

3. **Summary** (2 copies) This should be a short summary of results and conclusions arranged in numbered paragraphs.

4. **Abstract** (2 copies) An abstract of about 100 words suitable for inclusion in an abstracting journal should be typed on a separate sheet, giving the author's name and address and the title of the paper.

5. **Figures** These should not be larger than A4 (206 x 294 mm) and may be in the form of original drawings, recorded tracings or high quality photographic prints made from them. Negative prints of kymograph tracings (black on white) should be provided, and both ordinates and abscissae should be calibrated. Lettering on figures should be inserted in pencil. Symbols which are to appear in figures or legends should be chosen from the following:

○ ● □ ■ △ ▲ ▽ ▼

Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction.

6. **Tables** These should not have more than 85 characters to a line (counting spaces between columns as 4 characters) and certainly not more than 110 characters to a line, unless absolutely unavoidable. **Figure legends** and **tables** should be typed on separate sheets of paper.

7. **References** These should be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the fourth edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should in addition, include the names of editors, the edition number, where appropriate, and the town of origin and name of publisher.

**Two page proofs** will be supplied, one of which may be retained by the authors. The other should be corrected immediately and returned to the Press Editor. Corrections should be kept to a minimum.

**Twenty-five reprints** will be supplied to the authors free of charge. Additional reprints can be purchased. A reprint order form, which should be returned promptly, will be sent out with the proofs. No reprints of abstracts are supplied.

**Short Communications** These may be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text and only one figure or table. The summary should be a single paragraph. An abstract and statement should be included.

# British Journal of Pharmacology

VOLUME 58 NUMBER 1 SEPTEMBER 1976

3 BUDNY, J., DOW, R.C., ECCLESTON, D., HILL, A.G. & RITCHIE, ISOBEL M. A comparison of D and L-tryptophan on the cerebral metabolism of 5-hydroxytryptamine and dopamine in the dog

9 COLLIER, H.O.J., HAMMOND, M.D. & SCHNEIDER, C. Effects of drugs affecting endogenous amines or cyclic nucleotides on ethanol withdrawal head twitches in mice

17 LUNDELL, L., SVENSSON, S.E. & NILSSON, G. Further studies on the mode of action of isoprenaline on gastric secretion in the conscious rat

27 TURNBULL, M.J. & WATKINS, J.W. Determination of halothane-induced sleeping time in the rat: effect of prior administration of centrally active drugs

37 DAVE, K.C. & DHATTIWALA, A.S. Adrenoceptors of the guinea-pig urinary bladder

43 HÖGLUND, ELIN, WESTLING, H. & WHITE, T. Circulatory effects of oral and subcutaneous administration in normal subjects of a new bronchodilator ibuterol, a pro-drug to terbutaline

47 GILLESPIE, J.S. & TILMISANY, A.K. The action of tetraethylammonium chloride on the response of the rat anococcygeus muscle to motor and inhibitory nerve stimulation and to some drugs

57 ROBERTS, FIONA & STEPHENSON, R.P. The kinetics of competitive antagonists on guinea-pig ileum

71 LULICH, K.M., MITCHELL, H.W. & SPARROW, M.P. The cat lung strip as an *in vitro* preparation of peripheral airways: a comparison of $\beta$-adrenoceptor agonists, autacoids and anaphylactic challenge on the lung strip and trachea

81 BEAL, A.M. The effect of high rates of vasopressin administration on renal potassium and sodium excretion during potassium loading in the sheep

89 BROADLEY, K.J. The release of a coronary vas dilator metabolite from the guinea-pig isolat perfused heart stimulated by catecholamine histamine and electrical pacing and by exposure anoxia

101 JHAMANDAS, K. & SUTAK, Morphine–naloxone interaction in the centr cholinergic system: the influence of subcortic lesioning and electrical stimulation

109 EVANS, R.H. & SMITH, J.W. The effect of ca cholamines on the influx of calcium and t development of tension in denervated mou diaphragm muscle

117 TAYLOR, M. Effects of L-tryptophan and methionine on activity in the rat

121 TODA, N. Influence of dopamine and n adrenaline on isolated cerebral arteries of the d

127 RICHARDSON, P.D.I. The actions of natu secretin on the small intestinal vasculature of t anaesthetized cat

137 BOREHAM, P.F.L. & WRIGHT, I. Hypotension in rabbits infected with *Trypa soma brucei*

141 CREESE, R. & MACLAGAN, JENNIFE Labelled decamethonium in cat muscle

149 BUNCE, K.T., PARSONS, M.E. & ROLLING NITA A. The effect of metiamide on acid secreti stimulated by gastrin, acetylcholine and dibuty cyclic adenosine 3′,5′-monophosphate in t isolated whole stomach of the rat

## SHORT COMMUNICATION

157 BROWN, D.A. & GARTHWAITE, HAYASHI, E. & YAMADA, S. Action surugatoxin on nicotinic receptors in the super cervical ganglion of the rat

Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge

CURRENT ISSUE

24

# British Journal of Pharmacology

NOT TO BE LENT OUT

October 1976

BRITISH HIGH COMMISSION.
BRITISH C[illegible] DIVISION.
[illegible] Sarani,
Calcutta-700016

5.7.4/1

# Edited for the British Pharmacological Society by

A.W. Cuthbert *(Chairman)* H.M. Adam *(Secretary)*
Margaret Day *(Press Editor)*
J.D. Stephenson *(Assistant Press Editor)*

A.M. Barrett
W.G. Bisset
T.B. Bolton
D.A. Brown
B.A. Callingham
T.J. Crow
F. de Matteis
C.T. Dollery
V. Eisen
E.W. Gill
A.F. Green
D.F. Hawkins
Margarethe Holzbauer
D.B. Hope
J.S. Kelly
G.M. Lees
G.P. Lewis
I.H.M. Main
E.K. Matthews
J.F. Mitchell
P.A. Nasmyth
M.W. Parkes
M. Sandler
A.P. Silverman
M.A. Simmonds
F.M. Sullivan
A. Ungar
R.A. Webster

Papers will be considered for publication on all aspects of drug action, including chemotherapy.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ. Authors should consult the instructions inside the back cover.

*The British Journal of Pharmacology* is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription £45.00 (USA & Canada $99. Rest of world $92). (Orders must be accompanied by remittance. Prices correct at time of publication.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

Enquiries concerning advertising space and rates should be addressed to: Advertisement Department, Macmillan Journals Limited, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Copyright © 1976 by the *British Journal of Pharmacology*. All rights of reproduction are reserved in respect of all papers, articles, illustrations, etc., published in this journal in all countries of the world.

Second class postage paid at New York, N.Y.
US Mailing Agent, Expeditors of the Printed Word, Ltd,
527 Madison Avenue, New York, NY 10022.

# British Journal of Clinical Pharmacology

The British Journal of Clinical Pharmacology is the authoritative channel of communication between clinical and academic research and that of the pharmaceutical industry. Published bi-monthly, the journal aims to encourage the cross-fertilisation of information and ideas, to help avoid the dangers of compartmentalisation.

BJCP contains original papers and reports on all aspects of drug action in man. These include the pre-clinical testing of drugs in normal volunteers and important clinical trials in patients, using the latest techniques.

Editorial provides informed comment and evaluation of papers in terms of contemporary clinical practice,while Letters to the editors allow immediate discussion of urgent observations and negative reports.

Recent papers include:
Physiological and psychological effects of ±-propranolol, +-propranolol and diazepam in induced anxiety, P. J. Tyrer & M. H. Lader.
Acute effect of antipyretic analgesics, alone or in combination with alchohol, on human psychomotor skills related to driving, M. Linnoila, T. Seppala & M. J. Mattila.
Effect of phenobarbitone on hepatic drug-metabolizing enzymes and urinary d-glucaric acid excretion in man, D. S. Lecamwasam, C. Franklin & P. Turner.

**Annual subscription £28.00 (USA and Canada $65.00, Rest of World $60.00)**
Orders must be accompanied by remittance. Prices correct at time of publication. Cheques should be made payable to: Macmillan Journals Ltd., Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

## Merrell International Research Center
## Strasbourg, France

### Pharmacologist

Applications are invited for the position of a senior pharmacologist to assume responsibility for continued preclinical development of new pharmacologic agents. The position involves further evaluation of compounds with already demonstrated biochemical or biologic activities in close cooperation with multidisciplinary groups engaged in basic research.

The candidate should have a Ph.D. or equivalent and extensive experience in laboratory evaluation of drug actions. Innovative ability in the design or adaptation of experimental methods is highly desirable. Knowledge of French would be helpful but is not mandatory.

Applications including a curriculum vitae should be directed to:

J. Koch-Weser, M.D.
**Centre de Recherche Merrell International**
16, rue d'Ankara
67000 Strasbourg, France

---

## British Journal of Pharmacology

British Journal of Pharmacology provides up-to-date research on all aspects of drug action essential to those involved in pharmacology. Ensure you receive this information each month by taking a subscription.

Subscription Dept, Macmillan Journals Ltd, Brunel Road, Basingstoke, Hants RG21 2XS, England.

Please enter me for a year's subscription to the BRITISH JOURNAL OF PHARMACOLOGY starting with the issue dated . . . . . . . . . . . .

I enclose payment of £45.00 (USA & Canada $99. Rest of world $92).

Prices correct at time of publication. Payment may be made in any currency at the current exchange rates. Orders can only be processed if accompanied by the correct remittance. Cheques should be made payable to Macmillan Journals Ltd.

Name . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Address . . . . . . . . . . . . . . . . . . . . . . . . . . . .
. . . . . . . . . . . . . . . . . . . . . . . . . . . .
. . . . . . . . . . . . . . . . . . . . . . . . . . . .
. . . . . . . . . . . . . . . . . . . . . . . . . . . .

Registered No 785998 England Registered Office: 4 Little Essex St., London WC2R 3LF

VOLUME 58(2) OCTOBER 1976

# British Journal of Pharmacology

165 COLINA-CHOURIO, J., McGIFF, J.C., MILLER, M.P. & NASJLETTI, A. Possible influence of intrarenal generation of kinins on prostaglandin release from the rabbit perfused kidney

173 BRIATICO, G., GUISO, G., JORI, A. & RAVAZZANI, C. Effects of contraceptive agents on drug metabolism in various animal species

183 STEINESS, EVA & VALENTIN, N. Myocardial digoxin uptake: dissociation between digitalis-induced inotropism and myocardial loss of potassium

189 KISIN, I.E. On the decrease of myocardial oxygen consumption induced by chloracyzine

193 COLLIER, H.O.J., McDONALD-GIBSON, WENDY J. & SAEED, S.A. Stimulation of prostaglandin biosynthesis by drugs: effects *in vitro* of some drugs affecting gut function

201 FERREIRA, S.H. & SMAJE, L.H. Bradykinin and functional vasodilatation in the salivary gland

211 APPERLEY, EIRA, HUMPHREY, P.P.A. & LEVY, G.P. Receptors for 5-hydroxytryptamine and noradrenaline in rabbit isolated ear artery and aorta

223 ABOULAFIA, JEANNINE, MENDES, GUN MIYAMOTO, MARIA E., PAIVA, A.C.M. PAIVA, THEREZINHA B. Effect indomethacin and prostaglandin on the smoo muscle contracting activity of angiotensin ar other agonists

229 DALE, M. MAUREEN, EVINÇ, AKGÜN VINE, PAULINE. The denervated cremast muscle of the guinea-pig as a pharmacologic preparation

239 BEVAN, P., BRADSHAW, C.M. & SZABAD E. The action of micro-electrophoretically appli L-3,4-dihydroxyphenylalanine (DOPA) on sing cortical neurones

247 CLANACHAN, A.S. & McGRATH, J.C. Effec of ketamine on the peripheral autonomic nervo system of the rat

253 SMITH, G.W. & SOMOVA, LILIANA I. Ren function and renal venous prostaglandin co centrations during different stages of experiment renal hypertension in the rat

261 KALSNER, S. The lack of effect of ox tetracycline on responses to sympathetic ner stimulation and catecholamines in vascular tissu

267P PROCEEDINGS OF THE BRITIS PHARMACOLOGICAL SOCIETY, Dunde 15–16 July, 1976

## PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY

University of Dundee, 15th–16th July, 1976

7P GARDNER, C.R. & PHILLIPS, S.W. Heterogeneity of inhibitory mechanisms in the nucleus accumbens and preoptic area of the rat

9P BAXTER, M.G., MILLER, A.A. & WHEATLEY, P.L. Comparative studies on the effects of metoclopramide and some known neuroleptics on the EEG of the conscious rat

0P HILL, R.C. & RÖMER, D. Comparison of the self-administration liabilities of morphine and pentazocine in the rhesus monkey

1P CHARLTON, K.G. & SUGRUE, M.F. Some effects of mazindol on the metabolism of monoamines in the rat brain

2P ARBUTHNOTT, G.W., GARCIA-MUNOZ, M., NICOLAOU, N.M., TULLOCH, I.F. & WRIGHT, A.K. (introduced by Ashcroft, G.W.). Is the striato-nigral pathway responsible for 'feed-back' control of dopamine release?

72P KRUK, Z.L. & ZARRINDAST, M.R. The effects of anorectic drugs on uptake and release of brain monoamines

74P ELFELLAH, M.S., MARSHALL, P.B. & TURNBULL, M.J. Effect of chronic pretreatment with sympathomimetic bronchodilator drugs on the responsiveness of guinea-pig lung adenylate cyclase *in vitro*

75P COWAN, A., DETTMAR, P.W. & WALTER, D.S. The effect of acute doses of buprenorphine on concentrations of homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) in the rat forebrain

75P ALGATE, D.R. & LEACH, G.D.H. The stimulated release of dopamine-$\beta$-hydroxylase from intact organs of normo- and hypertensive rats

76P FOZARD, J.R. & MOBAROK ALI, A.T.M. Evidence for tryptamine receptors on cardiac sympathetic nerves

78P IDOWU, O.A. & ZAR, M.A. Inhibitory effect of clonidine on a peripheral adrenergic synapse

79P GEORGE, A.J. & JONES, G.T. Studies on monoamine oxidase in rat spinal cord

280P DEHPOUR, A.R., GHAFFARPOUR, F., HOSSEINZADEH, K. & KHOYI, M.A. (introduced by Michal, F.) Effect of denervation and cocaine on the response of isolated rat vas deferens to noradrenaline and methoxamine

280P SUMMERS, R.J. & TILLMAN, JANET. The effects of AH 5158 on metabolism of $^3$H(—)noradrenaline released from the cat spleen by nerve stimulation

281P CORBETT, A.D., MAIN, BARBARA E., MUIR, T.C. & TEMPLETON, D. The receptors involved in catecholamine mediated growth and secretion in rat parotid gland

282P FINCH, L. & HICKS, P.E. Studies on the marked antihypertensive properties of indapamide (SE 1520) in rats and cats

283P BOWMER, C.J. & LINDUP, W.E. Effect of endogenous metabolites on the binding of *o*-methyl red to human serum albumin

284P BENNETT, P.N., DAVIES, D.S. & HAWKESWORTH, GABRIELLE M. *In vivo* absorption studies with paraquat and diquat in the dog

285P TURNBULL, M.J. & WATKINS, J.W. Change in sensitivity to halothane in the rat following administration of sodium barbitone in the drinking water

286P KIRBY, MARILYN J. & TURNER, P. Effect of some receptor antagonists on fenfluramine-induced glucose uptake into the isolated rat hemidiaphragm

287P JETLEY, M. & WESTON, A.H. Some effects of D600, nifedipine and sodium nitroprusside on electrical and mechanical activity in rat portal vein

288P DALE, M. MAUREEN & MUID, R. A simple *in vitro* preparation of mammalian tissue exhibiting properties of slow tonic skeletal muscle

289P DAVEY, A., MARRIOTT, A. & STOCKHAM, M.A. Fitting a general sigmoid model to pharmacological response curves

290P SANGER, G.J. & WATT, A.J. A postsynaptic action of prostaglandin $E_1$ on sympathetic responses in guinea-pig ileum

291P BOURNE, R.C., FRITH, C.D., OWEN, F. & RILEY, G.J. (introduced by Crow, T.J.). The effect of subcutaneous injections of adrenaline on platelet MAO activity

292P BACH, CATHERINE S. & JASANI, M.K. Role of lymphocytes in accumulation of fibrin in rabbit skin homografts

294P BLAKELEY, A.G.H. & NICOL, C.J.M. Accumulation of biogenic amines by rabbit erythrocytes

295P MITCHELL, SHEILA M., POYSER, N.L. & WILSON, N.H. Effect of *p*-bromophenacyl bromide, an inhibitor of phospholipase $A_2$, on prostaglandin production by the guinea-pig uterus

296P BUNTING, S., HIGGS, G.A. MONCADA, S. & VANE, J.R. Generation of thromboxane $A_2$-like activity from prostaglandin endoperoxides by polymorphonuclear leukocyte homogenates

297P WESTWICK, J. (introduced by Lewis, G.P.). The effect of pulmonary metabolites of prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ on ADP-induced aggregation of human and rabbit platelets

298P GORDON, J.L. & MacINTYRE, D.E. Stimulation of platelets by bis-enoic prostaglandins

299P GORDON, J.L., MacINTYRE, D.E. & McMILLAN, R.M. Malondialdehyde production and the release reaction in rat blood platelets: inhibition by aspirin and indomethacin *ex vivo*

300P GORDON, J.L. & OVERMAN, H.J. Transport of 5-hydroxytryptamine by rat and human platelets

301P JACKSON, D.M. & RICHARDS, I.M. A further action of sodium cromoglycate

302P GIBSON, A., POLLOCK, D. & SPENCE, J.C. The effects of corticosteroids on the responses of the anococcygeus and gastrocnemius muscles to nerve stimulation in the pithed rat

303P GOADBY, P. & LITTLE, M. ANN. The action of metiamide in anaphylaxis *in vivo* in the guinea-pig

304P REGOLI, D., ST-LOUIS, J. & the late PARK, W.K. Factors regulating the time-course of the relaxation of rabbit aorta strips after contraction by angiotensin II

305P GIBSON, A. & JAMES, T.A. $K^+$-ind relaxations of the rat anococcygeus muscle

305P FOSTER, R.W. How can one set multiple ch examinations of equal difficulty from year to y

306P HOULT, J.R.S. Comparisons of performanc pharmacology examinations using multiple ch and essay questions

307P FOY, J.M. Learning from and attitudes to rou tape-slide teaching

308P FOY, J.M. & SENIOR, JUDITH. The teachin pharmacology to undergraduate pharm students

309P MARSHALL, P.B. & STEVENSON, I.H. Car patterns in science graduate pharmacologists

310P FIRRELL, J.C., PECK, M.J. & WILLIAMS, (introduced by Lewis, G.P.). The polymo transfer reaction: a model system for the study acute inflammation

311P COTTNEY, J., LEWIS, A.J. & NELSON, D Arachidonic acid-induced paw oedema in the

312P LUSCOMBE, D.K. & NICHOLLS, P.J. Cl experiment with student volunteers illustrating t influence of formulation on absorption of aspi

312P LISCOMBE, D.K. & NICHOLLS, P. Intersubject variability of sulphadimidi acetylation in student volunteers

313P HOULT, J.R.S. Films in pharmacology teachi

314P D'MELLO, A. & KRUK, Z.L. Effecti pharmacological demonstrations to 100 student

314P LEACH, G.D.H. Developments in the use television for pharmacology teaching

315P COX, B., FOZARD, J.R. & SMALL, R.C. Slid tape programmes in the teaching of pharmacolog

315P FOY, J.M. & HOPKINS, D.J. Autonomic dru and the eye

316P FOY, J.M. & SENIOR, JUDITH. Teachin methods in pharmacology

316P LEACH, G.D.H. Langendorff mammalian hear perfusion

5P FOY, J.M. & McCURRIE, J.R. The blood pressure and respiration of the anaesthetized cat

7P BLACK, A. KOBZA, GREAVES, M.W., HENSBY, C.N. & PLUMMER, N.A. A new method of obtaining human skin inflammatory exudate for pharmacological analysis

317P WEIR, R.D., MOIR, D.C., ERSKINE, Z.G., GALLON, S.C., DAVIDSON, J.F., CROOKS, J. & CHRISTOPHER, L.J. Aberdeen-Dundee medicines evaluation and monitoring group

318P TELLA, A. Studies on *Azadiracta indica* in malaria

*Br. J. Pharmac.* (1976), **58**, 165–172

# POSSIBLE INFLUENCE OF INTRARENAL GENERATION OF KININS ON PROSTAGLANDIN RELEASE FROM THE RABBIT PERFUSED KIDNEY

J. COLINA-CHOURIO, J.C. McGIFF, M.P. MILLER & A. NASJLETTI
Department of Pharmacology, University of Tennessee, Center for the Health Sciences,
800 Madison Avenue, Memphis, Tennessee 38163, U.S.A. and
Department of Pharmacology, The Medical College of Wisconsin, Milwaukee, Wisconsin 52322, U.S.A.

1 The effects of bradykinin and kininogen on renal prostaglandin release were studied in rabbit isolated kidneys perfused with oxygenated Krebs solution.

2 The concentration of prostaglandin-like material in kidney effluent was determined by bioassay after extraction of the samples with organic solvents. In 7 experiments the samples were assayed after separation of prostaglandins E and F by thin layer chromatography.

3 Addition of bradykinin to the perfusing fluid increased the venous and urinary effluxes of prostaglandin E-like substance by sixfold and fivefold, respectively, but efflux of prostaglandin F-like material was unaffected.

4 Addition of kininogen to the perfusing fluid augmented the venous and urinary release of prostaglandin E-like substances by fifteenfold and ninefold respectively and caused a twofold increase in the efflux of prostaglandin F-like material into the venous effluent.

5 Aprotinin, a kallikrein inhibitor, reduced the prostaglandin releasing action of kininogen but not of bradykinin. In contrast, inhibition of prostaglandin synthesis by indomethacin suppressed the release of prostaglandin evoked by either bradykinin or kininogen.

6 This study suggests that augmented release of prostaglandins in response to kininogen is a consequence of renal generation of kinins. Thus, changes in the intrarenal activity of the kallikrein-kinin system may modulate renal prostaglandin release.

## Introduction

Kinins and prostaglandins of the E series share the ability to produce renal vasodilatation, diuresis, and natriuresis, and thereby may contribute to the regulation of blood pressure, renal blood flow and salt-water excretion (Adetuyibi & Mills, 1972; Margolius, Geller, de Jong, Pisano & Sjoerdsma, 1972; McGiff, Crowshaw & Itskovitz, 1974; Nasjletti, Colina-Chourio & McGiff, 1975a).

The demonstration that close arterial infusion of bradykinin augments the release of E prostaglandins into renal venous blood (McGiff, Terragno, Malik & Lonigro, 1972) suggested a relationship between blood levels of kinins and renal prostaglandin release. A relationship between kinins generated intrarenally and prostaglandin release depends on the ability of the kinin to reach sites of prostaglandin synthesis. Renal prostaglandin synthesizing enzymes occur predominantly in the medulla (Larsson & Änggard, 1973) while kallikrein is located in the cortex Nustad, 1970). The present experiments were undertaken to investigate the influence of intrarenal generation of kinins on the release of renal prostaglandins.

## Methods

### *Kidney perfusion*

Male New Zealand white rabbits (2.5–3.0 kg), maintained on a normal diet, were anaesthetized with sodium pentobarbitone (25 mg/kg i.v.) and injected with heparin (250 units/kg i.v.). After opening the abdomen, the left kidney was isolated and perfused as described by Regoli & Gauthier (1971). Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2.6H_2O$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ $7H_2O$ 1.17, dextrose 8.4 and $NaHCO_3$ 25.0 was aerated with 95% $O_2$ and 5% $CO_2$ and maintained at 37°C; it was delivered as pulsatile flow at constant rate (10 ml/min) by a roller pump. The ureter and renal vein of the perfused kidney were cannulated with polyethylene tubing and the perfusion fluid was not recirculated. A pressure transducer (Statham P23DB) was connected to a side arm of the arterial cannula and the perfusion pressure was recorded on a polygraph (Hewlett-Packard). Mean renal perfusion pressure, initially high (range 120–160 mmHg), fell rapidly to a steady lower level ($87 \pm 4$ mmHg) within 3–5 min of starting perfusion. The volume of the urinary effluent averaged $0.96 \pm 0.08$ ml/min during the first 10 min of perfusion indicating that about 10% of the perfusing fluid was excreted. The concentration of sodium, measured by flame photometry, was identical in venous and in urinary effluents. These observations are consistent with the demonstration by Regoli & Gauthier (1971) that the Krebs-perfused rabbit kidney does not reabsorb sodium nor does it concentrate urine. Ten min after starting perfusion the experiments were begun and the kidneys were perfused for a further 60 to 70 minutes. Venous and urinary effluents were collected for three or four different periods of 10 min each separated by 15 min intervals during which neither bradykinin nor kininogen were present in the perfusing fluid. The first period was used as a control while during the second and third periods bradykinin (1–10 ng/ml) or kininogen (0.5–5 mg/ml) were alternatively added to the perfusion medium. In some experiments the third period was used as another control. When the effects of indomethacin (5 µg/ml) or aprotinin (250 KIE/ml) were examined, these agents were added to the perfusion fluid before starting the perfusions and maintained throughout. To investigate time-related changes in prostaglandin effluxes, we perfused kidneys with Krebs solution alone for 70 min and collected samples of venous and urinary effluents at 10 min intervals. Kallikrein activity was measured in these samples to assess the ability of the perfused kidney to release an active kinin forming enzyme.

### *Drugs*

The following drugs were used: synthetic bradykinin (BRS 640, Sandoz Pharmaceuticals); indomethacin (Merck, Sharp and Dohme; aprotinin (Trasylol, Bayer) and pentobarbitone sodium (Abbott). Drugs were prepared in Krebs solution immediately before use.

### *Preparation of kininogen*

Canine plasma kininogen was prepared in our laboratory as follows: blood from pentobarbitone anaesthetized dogs (30 mg/kg, i.v.) was collected in plastic bottles containing enough sodium citrate and exadimethrine to establish concentrations of 3.1 mg/ml and 0.4 mg/ml respectively. After centrifugation (1500 *g*, 30 min, 4°C) and addition of disodium ethylenediaminetetraacetate (EDTA) (0.03 M) the mixture was kept at 62°C for 2 h and centrifuged. The supernatant was cooled to 2–4°C and ammonium sulphate was added (1.9 M) followed by continuous stirring (1 h) and centrifugation (1500 *g*, 30 min, 4°C). The precipitate was dissolved in a minimum amount of water and dialyzed against distilled water for 48 h (4 changes of water). Following centrifugation, the supernatant was acidified with 5 N HCl (pH 2.5, 32°C), neutralized (pH 7) 20 min later and freeze-dried. The kininogen content of this preparation was measured with a substrate exhaustion technique (Fasciolo, Espada & Carretero, 1963). One mg of the dry material was dissolved in 4.5 ml of 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl and incubated at 37°C for 15 min with either trypsin (0.5 mg) or dialyzed rabbit urine (0.2 ml). The enzymatic reaction was terminated by heating in boiling water (10 min) and the kinin content of the samples assayed by their vasodilator activity in the canine hind limb preparation (Nasjletti, Colina-Chourio & McGiff, 1975b), using synthetic bradykinin as the reference standard. One mg of the kininogen preparation had an activity equivalent to 140 ng of bradykinin. This amount of kinin precursor was contained in 0.03 ml of the original plasma. Contamination of the kininogen preparation with kinin forming enzymes was not likely since kinins were not generated when the protein material (10 mg) was dissolved in Krebs solution (1 ml) and incubated for 10 min at 37°C. Similar incubations, carried out in the presence of synthetic bradykinin (50 ng) resulted in complete recovery of the peptide, which indicates the absence of kininases. The kininogen preparation was free of renin substrate as indicated by its failure to liberate angiotensin when it was incubated with an excess of rabbit renin in the presence of angiotensinase inhibitors (Nasjletti & Masson, 1969). This was expected since inactivation of renin substrate by heating and acidification has been reported (Braun-Menéndez, Fasciolo, Leloir, Muñoz & Taquini, 1946).

### *Determination of prostaglandins*

The content of prostaglandin-like substances in venous and urinary effluents of perfused kidneys was determined as follows: samples were combined with five volumes of acetone, passed through a millipore filter and evaporated to 5–10 ml under reduced pressure at 35°C. This was followed by acidification (pH 3.5) with 1 N HCl and extraction (three times) with an equal volume of ethyl acetate. The combined ethyl acetate phases were evaporated to dryness and the residue taken up in 0.5–1 ml of saline (0.15 M NaCl) immediately before bioassay. Total prostaglandin-like activity (Table 1, Figures 1 and 2), representing a mixture of E and F prostaglandins, was bioassayed in terms of prostaglandin $E_2$ on rat stomach strips superfused with Krebs solution (Vane, 1969). Values are uncorrected for losses of 15% incurred during extraction.

In seven experiments (Figure 3) the ethyl acetate extract was taken up in a minimum amount of chloroform-methanol (4:1, v/v), applied as a band to a thin layer chromatography plate (Silica gel G plates, 0.5 mm thick, Brinkmann Instruments), and chromatographed using the solvent system chloroform: methanol: acetic acid (18:2:1, by volume). Marker plates, prepared by spotting 10 µg of authentic prostaglandins $E_2$ and $F_{2\alpha}$, were run concurrently with preparative plates. All plates were developed until the solvent had reached 16 cm from the origin. The marker plates were visualized by spraying with 10% phosphomolybdic acid in ethanol, followed by heating to 100°C for 15 minutes. The $R_F$ value of prostaglandins $E_2$ and $F_{2\alpha}$ were approximately 0.44 and 0.26 respectively. Zones on the preparative plate corresponding to the position of prostaglandins $E_2$ and $F_{2\alpha}$, the area in between them and the remainder of the plate divided into five 2 cm zones were scraped off and eluted with chloroform-methanol (4:1, v/v). Eluates were dried in $N_2$, reconstituted in 0.9% w/v NaCl solution (saline), and bioassayed for their content of prostaglandin E- and F-like materials using prostaglandins $E_2$ and $F_{2\alpha}$ respectively as reference standards. Efflux of prostaglandin-like material (flow rate × concentration) was expressed as nanograms of prostaglandins $E_2$ and $F_{2\alpha}$ equivalent per min (ng/min). The values were uncorrected for losses incurred on extraction and chromatographic purification. In six experiments, after addition of 50 ng of authentic prostaglandin $E_2$ to 50 ml of venous effluent obtained from a kidney perfused with Krebs solution containing indomethacin (5 µg/ml), we recovered 65 ± 6% (s.e.) of prostaglandin-like material. The concentration of prostaglandin-like substance in the samples was determined by bracket assay on rat stomach strips superfused with Krebs solution (Vane, 1969; Vane, 1971). To provide evidence in support of the tentative identification of prostaglandins in the samples, a rat colon and a chick rectum were included in the assay system. Thus, agreement between responses evoked by a sample and a prostaglandin standard on the rat stomach strip, rat colon and chick rectum, suggests that the myotropic activity of the sample is due to a prostaglandin (Vane, 1969). All three isolated organs were suspended in glass chambers and superfused in series with Krebs solution (3 ml/min, 37°C) (McGiff *et al.*, 1972).

Contractions of the tissues were measured by an isotonic transducer (model 356 Harvard Apparatus Company, Inc.) and recorded on a polygraph (model 7718, Hewlett-Packard). In the dose range used (0.5–5 ng), prostaglandin $E_2$ contracted the rat stomach strip and chick rectum, but had little effect on the rat colon; whereas prostaglandin $F_{2\alpha}$ contracted the rat colon and the chick rectum but had only one third the potency of prostaglandin $E_2$ in contracting the rat stomach strip. The crude ethyl acetate lipid extract contracted the three tissues in a manner similar, but not identical, to prostaglandin $E_2$. Thus, a dose of the lipid extract equivalent to prostaglandin $E_2$ in evoking contraction of the stomach strip, was slightly more effective than prostaglandin $E_2$ in contracting the rat colon. This discrepancy might be explained if prostaglandin E- and F-like material were present in the sample. This possibility was confirmed after thin layer chromatography of the crude lipid extract. Thus, the reconstituted eluate of the plate at zones corresponding to the migration of prostaglandins $E_2$ and $F_{2\alpha}$ standards contained material which contracted all three assay organs in a manner indistinguishable from prostaglandins $E_2$ and $F_{2\alpha}$ respectively. The demonstration of agreement between the substances isolated from the ethyl acetate extract and authentic prostaglandins $E_2$ and $F_{2\alpha}$ in terms of thin layer chromatographic behaviour and myotropic effects on three smooth muscle preparations is compatible with the interpretation that the myotropic activity of the lipid extract is due to prostaglandins. Release of prostaglandins $E_2$ and $F_{2\alpha}$ by the rabbit kidney has been established unequivocally by Davis & Horton (1972) using mass spectrometric analysis.

All prostaglandins assays were performed in duplicate; the difference between duplicate assays of 45 samples averaged 13.5 ± 2.5% (s.e. mean).

### *Determination of kallikrein*

Kallikrein activity in venous and urinary effluents was determined by the method of Marin-Grez & Carretero (1972), which is based on the determination of the amount of kinins formed when an aliquot of sample is incubated with an excess of kininogen. The lyophilized substrate (500 mg) was dissolved in 10 ml of a solution containing 0.15 M NaCl, 0.001 M 1–10 phenanthroline and 0.003 M EDTA. Each incubation mixture contained an aliquot of either venous (1 ml) or urinary (0.2 ml) effluents, 1 ml of kininogen solution and 0.15 M NaCl to a final volume of 3 ml. After incubation for 20 min at 37°C and pH 7.5, the mixture was acidified (pH 5–5.5) and heated in a boiling water bath for 5 minutes. Following centrifugation, the supernatant was neutralized (pH 7.0) and assayed in the canine hind limb preparation with synthetic bradykinin as reference standard (Nasjletti, *et al.*, 1975b). Kallikrein activity was expressed as nanograms of bradykinin equivalents released per millilitre of sample per min of incubation ($ng\ ml^{-1}\ min^{-1}$). Kallikrein release (flow rate × kallikrein activity), was expressed as nanograms of bradykinin equivalents per min (ng/min). The specificity of the kallikrein assay was assessed by the demonstration that addition of aprotinin (50 KIE) to mixtures of kininogen and urinary effluents (0.2 ml), blocks the generation of kinins. All the results were expressed as mean ± standard error (s.e.). Statistical significance was determined by Student's $t$-test based on unpaired or paired observation (Steel & Torrie, 1960). A $P$ value of 0.05 or less was considered statistically significant.

## Results

In nine isolated kidneys perfused for 70 min with Krebs solution only, the mean release of prostaglandin-like substances into the venous and urinary effluents at the start of perfusion was $1.9 \pm 0.8$ and $2.0 \pm 0.6$ ng of prostaglandin $E_2$ equivalents per min respectively and did not differ significantly from the values obtained in three subsequent observation periods (Table 1). Release of kallikrein into the urinary effluent was nearly constant throughout the perfusion but release into the venous effluent was detected in only two out of eight experiments (Table 1).

Addition of bradykinin to the perfusing fluid to establish concentrations of 1 ng/ml and 10 ng/ml, increased the venous efflux of prostaglandin-like substances from $1.7 \pm 0.6$ ng/min and $1.3 \pm 0.3$ ng/min to $3.6 \pm 0.7$ ng/min ($P < 0.02$) and $9.9 \pm 2.0$ ng/min ($P < 0.02$) respectively (Figures 1 and 2). Concomitantly, the urinary efflux of prostaglandin-like substances increased from $1.6 \pm 0.4$ ng/min and $1.3 \pm 0.5$ ng/min to $3.7 \pm 1.2$ ng/min ($P < 0.05$) and $10.4 \pm 3.4$ ng/min ($P < 0.025$) in response to 1 and 10 ng/ml of bradykinin respectively (Figures 1 and 2). Fifteen minutes after termination of bradykinin infusion (1 ng/ml), effluxes of prostaglandins into venous ($1.1 \pm 0.1$ ng/min) and urinary ($1.8 \pm 0.6$ ng/min) effluents were not different from control ($1.0 \pm 0.2$ ng/min, $P > 0.6$ and $1.9 \pm 0.4$ ng/min, $P > 0.8$, respectively; 4 experiments). Additions of kininogen to the perfusing fluid to establish concentrations of 0.5 mg/ml and 5 mg/ml, increased the

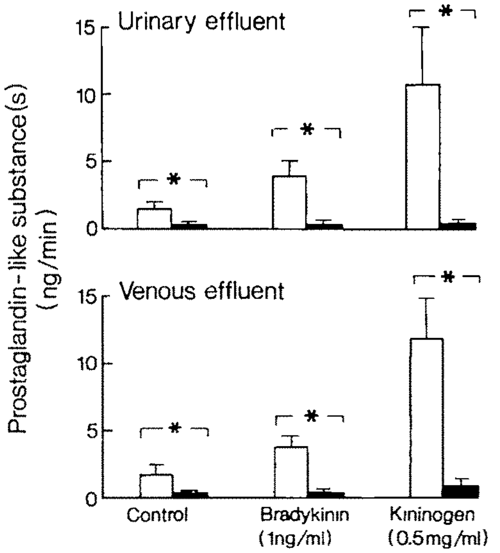


**Figure 1** The effect of bradykinin and kininogen on prostaglandin release in the presence (closed columns, $n = 6$) and in the absence (open columns, $n = 11$) of indomethacin (5 µg/ml). Vertical lines indicate the s.e. mean. Probability level (non-paired Student's $t$-test): $^*P < 0.01$.

**Table 1** Time course of prostaglandin and kallikrein release from rabbit perfused kidneys

| *Perfusion time (min)* | *0–10* | *20–30* | *40–50* | *60–70* |
|---|---|---|---|---|
| Prostaglandin release into the venous effluent (ng/min) ($n = 9$) | 1.9 ± 0.8 | 2.1 ± 0.4* | 2.1 ± 0.4* | 1.9 ± 0.4* |
| Prostaglandin release into the urinary effluent (ng/min) ($n = 9$) | 2.0 ± 0.6 | 1.6 ± 0.3* | 1.3 ± 0.3* | 1.7 ± 0.3* |
| Kallikrein release into the venous effluent (ng/min) ($n = 8$) | 1.9 ± 1.4 | 0.3 ± 0.3* | 1.2 ± 0.9* | 7.4 ± 7.4* |
| Kallikrein release into the urinary effluent (ng/min) ($n = 8$) | 82.5 ± 16.0 | 74.7 ± 10.6* | 81.5 ± 11.8* | 90.1 ± 11.1* |

Results are means ± s.e. mean; $n$ = number of experiments.
Significance of difference from controls (paired Student's $t$-test): $^*P > 0.1$.

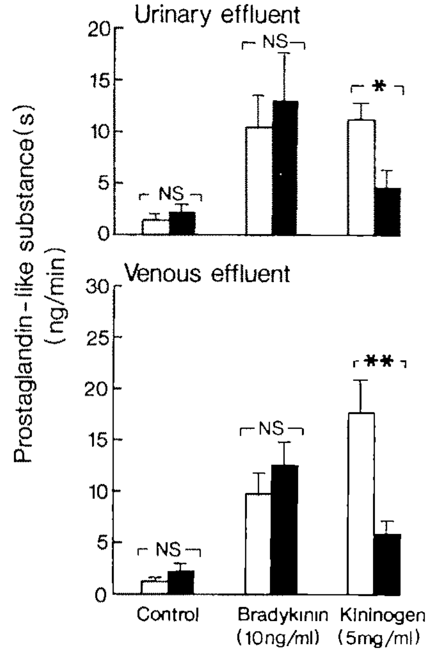


**Figure 2** The effect of bradykinin and kininogen on prostaglandin release in the presence (closed columns, $n=8$) and in the absence (open columns, $n=6$) of aprotinin (250 KIE/ml). Vertical lines indicate the s.e. mean. Probability level (non-paired Student's $t$-test): NS=$P>0.05$; *$P<0.02$; **$P<0.001$.

venous efflux of prostaglandin-like substances from 1.7 ± 0.6 ng/min and 1.3 ± 0.3 ng/min to 10.5 ± 3.0 ng/min ($P<0.01$) and 17.8 ± 2.8 ng/min ($P<0.005$) respectively (Figures 1 and 2). Concomitantly, the urinary efflux of prostaglandin-like material increased from 1.6 ± 0.4 ng/min and 1.3 ± 0.5 ng/min to 10.3 ± 4.4 ng/min ($P<0.05$) and 11.6 ± 1.7 ng/min ($P<0.001$) in response to the low (0.5 mg/ml) and high (5 mg/ml) concentration of kininogen respectively (Figures 1 and 2). Fifteen minutes after termination of the kininogen infusion (0.5 mg/ml), effluxes of prostaglandin into venous (1.7 ± 0.4 ng/min) and urinary (2.2 ± 1.1 ng/min) effluents were not different from control (1.5 ± 0.3 ng/min, $P>0.7$ and 1.7 ± 0.4 ng/min, $P>0.3$ respectively; 4 experiments).

Addition of indomethacin (5 µg/ml) to the perfusion fluid reduced the basal release of prostaglandin into venous and urinary effluents by 92% ($P<0.01$) and 87% ($P<0.01$) respectively and prevented the release evoked by bradykinin (1 ng/ml) and kininogen (0.5 ng/ml) (Figure 1). The kallikrein inhibitor aprotinin (250 KIE/ml) did not alter the basal or the bradykinin-induced efflux of prostaglandins but reduced the effect of kininogen (5 mg/ml) on venous and urinary release of prostaglandin-like substances by 79% ($P<0.001$) and 77% ($P<0.02$) respectively (Figure 2).

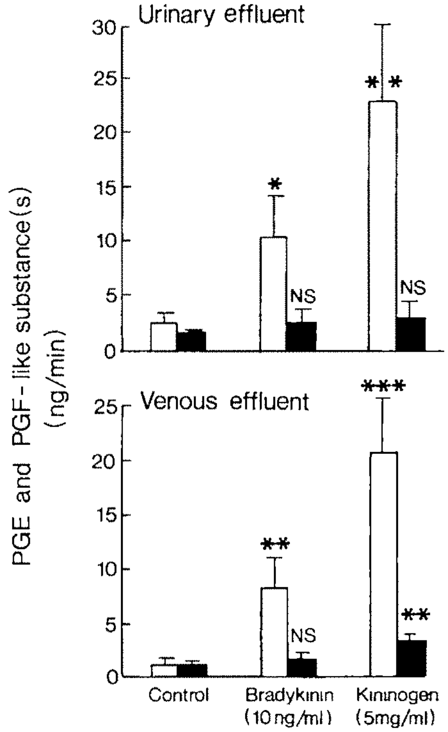


**Figure 3** The effect of bradykinin and kininogen on the release of prostaglandin E (PGE, open columns) and prostaglandin F (PGF, closed columns)-like substances from the perfused rabbit kidney ($n=7$). Vertical lines indicate the s.e. mean. Significance of difference from controls (paired Student's $t$-test): NS=$P>0.05$; *$P<0.05$; **$P<0.025$; ***$P<0.01$.

Figure 3 illustrates the effects of bradykinin (10 ng/ml) and kininogen (5 mg/ml) on renal release of prostaglandin E- and F-like materials. Bradykinin did not affect the release of F prostaglandins but increased the venous and urinary efflux of prostaglandin E-like substances from 1.3 ± 0.5 and 2.6 ± 0.9 ng/min to 8.3 ± 2.7 ng/min ($P<0.025$) and 10.3 ± 3.8 ng of prostaglandin $E_2$ equivalents per min ($P<0.05$) respectively. Similarly, addition of kininogen to the perfusion fluid increased the release of prostaglandin E into venous and urinary effluents from 1.3 ± 0.5 ng/min and 2.6 ± 0.9 ng/min to 20.6 ± 5.4 ng/min ($P<0.01$) and 23.1 ± 7.5 ng/min ($P<0.025$) respectively. This was associated with a small increase in the release of prostaglandin F into the venous effluent from 1.2 ± 0.3 to 3.3 ± 0.6 ng of prostaglandin $F_{2\alpha}$ equivalents per min ($P<0.025$).

In all experiments, neither bradykinin nor kininogen affected the renal perfusion pressure (87 ± 4 mmHg) which remained constant until the experiments were terminated.

## Discussion

Renal prostaglandins are synthesized predominantly in the renal medulla (Larsson & Ånggard, 1973) and released into renal venous blood (McGiff, Crowshaw, Terragno & Lonigro, 1970) and urine (Frolich, Wilson, Sweetman, Smigel, Niew, Carr, Watson & Oates, 1975). Our experiments show that addition of bradykinin to the perfusion fluid does not affect renal perfusion pressure but increases the renal effluxes of prostaglandins by selectively increasing the release of prostaglandin E compounds. These results support the findings of McGiff *et al.* (1972) who showed that intrarenal arterial infusions of bradykinin, but not of eledoisin, augment the concentration of prostaglandin E-like substance in the renal venous blood of dogs. We cannot explain the failure of bradykinin to produce vasodilatation in Krebs perfused kidneys. In a similar preparation, Needleman, Kauffman, Douglas, Johnson & Marshall (1973) observed slight vasodilatation in response to bolus injections (100 ng) of bradykinin. The discrepancy may be due to the differences in dosage and in the administration of the peptide.

Our demonstration that blockade of prostaglandin synthesis with indomethacin (Vane, 1971) causes an immediate reduction of prostaglandin efflux is consistent with the view that there is no appreciable storage of prostaglandins in the kidney (Ånggard, Bohman, Griffin, Larsson & Maunsbach, 1972). Therefore, the increased effluxes of renal prostaglandins in response to bradykinin may be accounted for by enhanced release of newly synthesized prostaglandins. In the present experiments a crude kininogen preparation, devoid of renin substrate and of kinin forming enzymes, was shown to share with bradykinin the ability to increase renal prostaglandin efflux by selectively enhancing the release of E prostaglandins. This observation and the finding of a kinin forming enzyme in the urinary effluent of perfused kidneys (Roblero, Croxatto, Corthorn, Garcia & De Vito, 1973) raise the possibility that the prostaglandin releasing action of kininogen is mediated by kinins liberated by kallikrein from the protein substrate. The demonstration that aprotinin, an inhibitor of renal and of urinary kallikrein (Nustad, 1970) reduced the effect of kininogen on prostaglandin release supports this view. Alternative explanations, such as direct effects of either kininogen or other plasma proteins contained in the substrate preparation on the synthesis, release and inactivation of renal prostaglandins are improbable since such effects are not likely to be impaired by aprotinin. Moreover, a direct impairment by the kallikrein inhibitor of the synthesis, release and inactivation of prostaglandins is unlikely since in our experiments aprotinin affected neither the basal effluxes of prostaglandins nor the augmented release evoked by bradykinin. Finally, the possibility that plasmin, an aprotinin-inhibited kininogenase whose precursor appears to originate in the kidney (Vogel & Werle, 1968; Highsmith & Kline, 1973) liberated kinins in our experiments is unlikely since this enzyme is devoid of kininogenase activity when acting upon heated kininogen (Vogt, 1964). Kinins generated intrarenally may also affect the release of renal prostaglandins in intact animals. Thus, kallikreins synthesized in the kidney cortex and presumably secreted in urine at the level of the distal tubules, may act on kininogen to produce kallidin (lysylbradykinin) which in turn could reach the sites of prostaglandin synthesis in the renal medulla via the collecting ducts (Nustad, Kirsten Vaaje & Pierce, 1975; Scicli, Carretero, Hampton & Oza, 1975). This possibility is endorsed by the demonstration in urine of free kinins which originate intrarenally (Miwa, Erdos & Seki, 1968; Nasjletti *et al.*, 1975a) and by the occurrence of kininogen in the kidney (Sardesai, 1968; Werle & Zach, 1970). Moreover, we have observed that rats receiving daily injections of deoxycorticosterone (5 mg) for two weeks have an augmented urinary excretion of kallikrein and of prostaglandin E-like substances and that these effects are prevented by simultaneous treatment with aprotinin (100,000 KIU) for four days (Colina-Chourio, McGiff & Nasjletti, unpublished observation).

Release of renal prostaglandins in intact animals is continuous and may influence renal blood flow (Lonigro, Itskovitz, Crowshaw & McGiff, 1973), sodium excretion (Lee & Attallah, 1974), renal vascular reactivity (Aiken & Vane, 1973), and ultimately blood pressure (Colina-Chourio, McGiff & Nasjletti, 1975). Similarly, kinins generated intrarenally appear to be involved in the regulation of renal blood flow and salt-water excretion (Nasjletti *et al.*, 1975a; Adetuyibi & Mills, 1972; Marin-Grez, Cottone & Carretero, 1972). Contribution of prostaglandins to some of the renal actions of kinins has recently been suggested. Thus, in studies using canine isolated blood perfused kidneys, the effects of bradykinin on renal blood flow and free-water clearance were reduced by an inhibitor of prostaglandin synthesis (McGiff, Itskovitz & Terragno, 1975). These observations and the present study, suggesting a relationship between intrarenal generation of kinins and renal prostaglandin release, support the proposal that a functional coupling of kinins and prostaglandins within the kidney may be an important element in the regulation of renal blood flow and salt-water excretion. However, this conclusion should be considered provisional until studies in intact animals are available.

We wish to thank Cheri Fry, Jo Ann Early, Mary Lou Elward and Debbie Mitchell for their assistance. We wish also to thank Dr Gert L. Haberland, Farbenfabriken Bayer

AG, West Germany for the supply of Trasylol. This work was supported by U.S. Public Health Service Grant HL 15791, by American Heart Association Grant 73720 and by the Tennessee Heart Association. J.C.-C. is the recipient of a fellowship from the University of Zulia, Maracaibo, Venezuela. J.C.M. is a Burroughs Wellcome Fund Scholar in Clinical Pharmacology. A.N. is the recipient of National Heart and Lung Institute Research Career Development Award 1 KO4 HL 00163. Please send reprint requests to A.N.

## References

ADETUYIBI, A. & MILLS, I.H. (1972). Relation between urinary kallikrein and renal function, hypertension, and excretion of sodium and water in man. *Lancet*, **II**, 203–207.

AIKEN, J.W. & VANE, J.R. (1973). Intrarenal prostaglandin release attenuates the renal vasoconstrictor activity of angiotensin. *J. Pharmac. exp. Ther.*, **184**, 678–687.

ÄNGGÅRD, E., BOHMAN, S.O., GRIFFIN, J.E., LARSSON, C. & MAUNSBACH, A.B. (1972). Subcellular localization of the prostaglandin system in the rabbit renal papilla. *Acta physiol. scand.*, **84**, 231–246.

BRAUN-MENÉNDEZ, E., FASCIOLO, J.C., LELOIR, L.F., MUÑOZ, J.S. & TAQUINI, A.C. (1946). In *Renal Hypertension* p. 132. Charles C. Thomas: Springfield, Illinois.

COLINA-CHOURIO, J., McGIFF, J.C. & NASJLETTI, A. (1975). Development of high blood pressure following inhibition of prostaglandin synthesis. *Fedn Proc.*, **34**, 368 (abstract).

DAVIS, H.A. & HORTON, E.W. (1972). Output of prostaglandins from the rabbit kidney, its increase on renal nerve stimulation and its inhibition by indomethacin. *Br. J. Pharmac.*, **46**, 658–675.

FASCIOLO, J.C., ESPADA, J. & CARRETERO, O.A. (1963). The estimation of bradykininogen content of plasma. *Acta physiol. lat. amer.*, **13**, 215–220.

FROLICH, J.C., WILSON, T.W., SWEETMAN, B.J., SMIGEL, M., NIES, A.S., CARR, K., WATSON, J.T. & OATES, J.A. (1975). Urinary prostaglandins: Identification and origin. *J. clin. Invest.*, **55**, 763–770.

HIGHSMITH, R.F. & KLINE, D.L. (1973). Plasminogen restoration by the kidmey mediated by a plasma factor. *Am. J. Physiol.*, **225**, 1032–1037.

LARSSON, C. & ÄNGGÅRD, E. (1973). Regional differences in the formation and metabolism of prostaglandins in the rabbit kidney. *Eur. J. Pharmac.*, **21**, 30–36.

LEE, J.B. & ATTALLAH, A.A. (1974). Response of intrarenal $PGA_2$ to salt loading in the rabbit. *Circulation Res.*, **34–35** (suppl. I), 75–82.

LONIGRO, A.M., ITSKOVITZ, H.D., CROWSHAW, K. & McGIFF, J.C. (1973). Dependency of renal blood flow on prostaglandin synthesis in the dog. *Circulation Res.*, **32**, 712–717.

MARGOLIUS, H.S., GELLER, R.G., DE JONG, W., PISANO, J.J. & SJOERDSMA, A. (1972). Urinary kallikrein excretion in hypertension. *Circulation Res.*, **30–31** (suppl. II) 125–131.

MARIN-GREZ, M., COTTONE, J. & CARRETERO, O.A. (1972). A method for measurement of urinary kallikrein. *J. appl. Physiol.*, **32**, 428–431.

McGIFF, J.C., CROWSHAW, K., TERRAGNO, N.A. & LONIGRO, A.J. (1970). Renal prostaglandins: Possible regulators of the renal actions of pressor hormones. *Nature, Lond.*, **227**, 1255–1257.

McGIFF, J.C., TERRAGNO, N.A., MALIK, K.U. & LONIGRO, A.J. (1972). Release of a prostaglandin E-like substance from canine kidney by bradykinin. *Circulation Res.*, **31**, 36–43.

McGIFF, J.C., CROWSHAW, K. & ITSKOVITZ, H.D. (1974). Prostaglandins and renal function. *Fedn Proc.*, **33**, 39–47.

McGIFF, J.C., ITSKOVITZ, H.D. & TERRAGNO, N.A. (1975). The actions of bradykinin and eledoisin in the canine isolated kidney: Relationships to prostaglandins. *Clin. Sci. & Mol. Med.*, **49**, 125–131.

MIWA, I., ERDOS, E.G. & SEKI, T. (1968). Presence of three peptides in urinary kinin (substance Z) preparations. *Life Sci.*, **7**, 1339–1343.

NASJLETTI, A., COLINA-CHOURIO, J. & McGIFF, J.C. (1975a). Disappearance of bradykinin in the renal circulation of dogs: Effects of kininase inhibition. *Circulation Res.*, **37**, 59–65.

NASJLETTI, A., COLINA-CHOURIO, J. & McGIFF, J.C. (1975b). Assay of kinins by their effects on canine femoral blood flow. *Proc. Soc. exp. Biol. Med.*, **150**, 493–497.

NASJLETTI, A. & MASSON, G.M.C. (1969). Effects of corticosteroids on plasma angiotensinogen and renin activity. *Am. J. Physiol.*, **217**, 1396–1400.

NEEDLEMAN, P., KAUFFMAN, A.H., DOUGLAS, J.R., JOHNSON, E.M. & MARSHALL, G.R. (1973). Specific stimulation and inhibition of renal prostaglandin release by angiotensin analogs. *Am. J. Physiol.*, **224**, 1415–1419.

NUSTAD, K. (1970). The relationship between kidney and urinary kininogenase. *Br. J. Pharmac.*, **39**, 73–86.

NUSTAD, K., KIRSTEN VAAJE & PIERCE, J.V. (1975). Synthesis of kallikreins by rat kidney slices. *Br. J. Pharmac.*, **53**, 229–234.

REGOLI, D. & GAUTHIER, R. (1971). Site of action of angiotensin and other vasoconstrictors on the kidney. *Can. J. Physiol., Pharmac.*, **49**, 608–612.

ROBLERO, J.S., CROXATTO, H.R., CORTHORN, J.H., GARCIA, R.L. & DE VITO, E. (1973). Kininogenase activity in urine and perfusion fluid of isolated kidney. *Acta physiol. lat. amer.*, **23**, 566–568.

SARDESAI, V.M. (1968). Determination of bradykinin in blood and bradykininogen in tissues. *Can. J. Physiol. Pharmac.*, **46**, 77–79.

SCICLI, A.G., CARRETERO, O.A., HAMPTON, A. & OZA, N.B. (1975). Kallikrein localization in the nephron by the stop-flow technique. *Fedn Proc.*, **34**, 378 (abstract).

STEEL, R.G.D. & TORRIE, J.H. (1960). *Principles and procedures of statistics*. New York, Toronto, London: McGraw-Hill Book Co., Inc.

VANE, J.R. (1969). Release and fate of vasoactive hormones in the circulation. *Br. J. Pharmac.*, **35**, 209–242.

VANE, J.R. (1971). Inhibition of Prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature, Lond.*, **231**, 232–235.

VOGEL, R. & WERLE, E. (1968). Kallikrein inhibitors. In *Handbook of Experimental Pharmacology*, Vol. 25, ed. Erdos, E.G., pp. 213–249. Berlin and Heidelberg: Springer-Verlag.

VOGT, W. (1964). Kinin formation by plasmin, an indirect process mediated by activation of kallikrein. *J. Physiol., Lond.*, **170**, 153–166.

WERLE, E. & ZACH, P. (1970). Verteilung von kininogen in serum and geweben bei ratten und andergen saugetieren. *Z. Klin. Chem. Klin. Biochem.*, **8**, 186–189.

*(Received August 29, 1975.*
*Revised April 10, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 173–181

# EFFECTS OF CONTRACEPTIVE AGENTS ON DRUG METABOLISM IN VARIOUS ANIMAL SPECIES

G. BRIATICO, G. GUISO, A. JORI & C. RAVAZZANI
Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62,
20157 Milan, Italy

1 The effect on liver microsomal enzyme activity of three steroid contraceptive drug (SCD) combinations was compared in rats, mice and guinea-pigs. Lynestrenol plus mestranol, norethisterone plus mestranol and norethynodrel plus mestranol were given orally for 4 consecutive days (acute treatment) or 30 days (chronic treatment) at various doses eliciting an experimentally controlled antifertility activity which varied in its extent.

2 In rats and mice all the combined treatments (with the exception of norethynodrel plus mestranol in mice) were active as inducers of liver microsomal enzymes. This induction seems to be mediated mainly by the progestogenic compounds. Oestrogens showed a very poor effect bordering on significance only in a few cases.

3 No effect on liver microsomal protein or cytochrome P 450 concentration was obtained after treatment with doses capable of increasing the microsomal enzyme activity.

4 The activity of the liver microsomal enzymes did not appear to be reduced immediately (2 h) after the last administration of the SCD given during 4 or 30 days.

5 Contraceptive treatments at doses capable of eliciting complete antifertility activity were inactive on liver microsomal enzyme activity in guinea-pigs.

## Introduction

It has been shown that both endogenous and exogenous steroids are metabolized in the liver endoplasmic reticulum by the same enzyme system responsible for the metabolism of foreign compounds (Conney, 1967). There are also extensive reports on the effects exerted by steroids on liver microsomal enzyme activity and on the interference by these hormones with the metabolism of some drugs. Particular attention was given in this respect to the steroid contraceptive drugs (SCD) in view of their widespread use together with other drugs in human therapy. Progesterone (Juchau & Fouts, 1966; Tephly & Mannering, 1968; Soyka & Long, 1972) and progestogen compounds (Soyka & Deckert, 1974; Tüttenberg, Hüthwohl, Kahl & Kahl, 1974) have been found to be competitive inhibitors for substrates metabolized by the mixed oxidases *in vitro*. More contradictory results have been obtained *in vivo*. Previous studies from this laboratory (Jori, Bianchetti & Prestini, 1969) indicated that chronic treatment with steroid contraceptives did not block, but rather increase the activity of liver microsomal enzymes in rats. Similar inducing effects were obtained by Juchau & Fouts (1966) and Rümke & Noordhoek (1969).

An increase in the metabolism of barbiturates after oestrogen and progestogen contraceptive treatment was also found in mice (Blackham & Spencer, 1969; Garg & Ahmad, 1974).

At variance with these results, Freudenthal & Amerson (1974) failed to show any induction after treatment with synthetic progestogens but on the contrary, found a weak inhibition of microsomal drug metabolism. An impairment of drug metabolism was also reported in women taking oral steroid contraceptives (Crawford & Rudofsky, 1966; O'Malley, Stevenson & Crroks, 1972).

It was therefore of interest to investigate the effect of some of the more widely used contraceptive drugs on liver microsomal enzyme activities under experimental conditions which reproduced the pattern of human use. Therefore, we have administered chronically for periods covering one or more oestrous cycles, combined oestrogens and progestogens in the ratios used for contraceptive medication and in doses able to produce an experimentally controlled antifertility activity in several animal species.

## Methods

### *Animals*

Female Charles River rats weighing 220 ± 10 g, female $CD_1$ mice weighing 25 ± 3 g, and female PIR bright/Z guinea-pigs weighing 350 ± 50 g were used. The rats and mice were housed in makrolon cages (6 per cage) and the guinea-pigs in steel rod cages (5 per cage). The animals were kept at room temperature (22°C) with relative humidity of 60% and with a controlled light cycle of 12 h (6 h 30 min to 18 h 30 min). Food and water were given *ad libitum*.

### *Drugs*

The following drug combinations were used: norethynodrel plus mestranol, norethisterone plus mestranol and lynestrenol plus mestranol.

The drugs were dissolved in corn oil, given orally for a period of 4 days (acute treatment) or 30 days (chronic treatment). In the case of guinea-pigs treatment lasted 32 days which is the length of two oestrous cycles.

The doses used in each treatment group are shown in the tables. Controls received a corresponding amount of corn oil. The oestrous cycle was controlled by means of vaginal smears. Animals were killed by decapitation 2 h (at 17 h 00 min) or 18 h (at 9 h 00 min) after the last SCD administration. The livers were removed and immediately frozen and stored at −20°C.

Enzymatic activity was measured on the 9000 *g* supernatant fraction using the experimental conditions described by Kato & Takanaka (1967).

The supernatant was incubated with glucose-6-phosphate (50 μmol), nicotinamide (50 μmol), NADP (1.5 μmol), $MgCl_2$ (25 μmol), sodium phosphate buffer pH 7.4 (0.2 M) and substrates (aniline (5 μmol) *p*-nitro-anisole (1,5 μmol) and aminopyrine (5 μmol)) to a total mixture volume of 5 ml. The matabolites formed, *p*-aminophenol, *p*-nitrophenol and 4-amino antipyrine respectively, were determined by the method of Gilbert & Golberg (1965).

Liver microsomes, used for the determination of proteins by the method of Lowry, Rosenbrough, Farr & Randall, 1951 and of cytochrome P 450 by the method of Omura & Sato (1964), were prepared by $Ca^{2+}$ precipitation by the method described by Schenkman & Cinti (1972).

The antifertility effect was measured by a bioassay as follows:

*Rats*: in the acute experiments (4 days = 1 oestrous cycle) the first dose of the test compound was given on the morning of pro-oestrus. At 16 h 00 min on the same day 2 females were caged with 1 fertile male. In the chronic experiments (30 days) female rats were caged with males on the 26th day of treatment. In both types of experiments, fertilization was confirmed by the presence of sperm in the vaginal smears. At autopsy, 10 days after the end of treatment, the uterine horns were examined for the number of implantation sites.

*Mice*: the same procedure as used with rats was followed for mice with the exception that no attempt was made to control their oestrous cycle or to confirm fertilitization by the presence of sperm in the vaginal smears.

*Guinea pigs*: female adult guinea-pigs (65 days old) were treated with the contraceptive agents for 33 days in order to cover two oestrous cycles. Treatment was started 10 days after checking the opening of the vaginal membrane. On the second day of treatment 2 females were caged with 1 male and left together for the whole 33 days period. No attempt was made to confirm fertilization by the presence of sperm in vaginal smears. At autopsy, 25 days after the last administration, uterine horns were examined and the number of implantation sites recorded.

Statistical analysis of the averages was carried out by Student's *t* test.

## Results

### *Antifertility activity of the contraceptive drugs in rats, mice and guinea-pigs*

In Table 1 the antifertility efficacy of the combined treatment with lynestrenol + mestranol, norethisterone + mestranol and norethynodrel + mestranol in the three animal species studied, is reported. These data are necessary to establish the significance of the steroid contraceptive drug dosages on the liver microsomal enzyme activity. It should be noted that the efficacy of the contraceptives with the exception of norethynodrel + mestranol, is similar in rats and mice after an acute treatment covering one oestrous cycle (4 days). In rats no significant difference appears between the chronic (30 days) and acute treatment; in mice the results indicate a higher activity of the combinations given chronically. In guinea-pigs all the contraceptive drugs tested appeared to be more effective than in rats and mice, although the 32 days chronic treatment covers only two complete oestrous cycles.

### *Liver microsomal activity in rats and mice during the oestrous cycle*

Vaginal smears were examined daily from untreated rats and mice for three consecutive oestrous cycles (12 days). In the experiments with the mice 2 males were confined in a wire basket in the cage containing 10 females to obtain more regular oestrous cycles (Whitten, 1957; Whitten, 1958). Animals with anomalous rhythms were eliminated, and those with regular four-day cycles were divided into three groups of 10 animals each and killed in pro-oestrus, oestrus and dioestrus respectively. No differences were observed during the various phases of the cycle on the metabolic activity of the liver microsomal enzymes *in vitro* or on the levels of cytochrome P 450 (nmol/mg protein 0.510 ± 0.005; 0.520 ± 0.008; 0.460 ± 0.054 in pro-oestrus, oestrus and dioestrus respectively in the rat). Therefore, the effect of the treatment with contraceptive steroids was compared in subsequent experiments with the activity of a control group without taking into consideration the oestrous state of the animals.

### *Effects of treatment with contraceptive drugs on liver microsomal enzyme activity in rats*

*Acute treatment.* The effect of a short period of treatment (4 days), corresponding to one oestrous cycle, with a large dose of lynestrenol (5 mg/kg) plus mestranol (0.3 mg/kg) or of norethisterone (4 mg/kg) plus mestranol (0.2 mg/kg) was studied in rats. Results of these experiments are shown in Table 2. No significant effect on microsomal enzyme activity was obtained in rats killed 18 h after the last administration of the first SCD combination, but treatment with the second combination in the same experimental conditions significantly increased the enzyme activity for the three substrates utilized.

**Table 1** Antifertility effects of contraceptive drugs on rats, mice and guinea-pigs

| *Treatment* | | *Number of implantation sites (% of controls)* | | | | |
|---|---|---|---|---|---|---|
| | | *Rat* | | *Mouse* | | *Guinea-pig* |
| *(mg/kg per day, orally)* | | *Days of treatment* | | | | |
| | | 4 | 30 | 4 | 30 | 32 |
| Lynestrenol<br>+ mestranol | (5)<br>(0.3) | 0 | 0 | 0 | 0 | 0 |
| Lynestrenol<br>+ mestranol | (2.5)<br>(0.15) | 45 | 33 | 6 | 0 | 0 |
| Lynestrenol<br>+ mestranol | (1.25)<br>(0.075) | 45 | 33 | 32 | 17.5 | 0 |
| Lynestrenol<br>+ mestranol | (0.625)<br>(0.037) | 60 | 68 | — | — | 10 |
| Norethisterone<br>+ mestranol | (4)<br>(0.2) | 1 | 0 | 9 | 6 | 0 |
| Norethisterone<br>+ mestranol | (2)<br>(0.1) | 25 | 10 | 27 | 9 | 0 |
| Norethisterone<br>+ mestranol | (1)<br>(0.05) | 39 | 37 | 48 | 10 | 0 |
| Norethynodrel<br>+ mestranol | (4)<br>(0.006) | 5 | 10 | 5 | 0 | 0 |
| Norethynodrel<br>+ mestranol | (2)<br>(0.03) | 31 | 44 | 8 | 1 | 0 |
| Norethynodrel<br>+ mestranol | (1)<br>(0.015) | 56 | 50 | 6 | 0 | 0 |

Groups of 15–20 animals were used for each dose. In control animals the following values were obtained: rats, pregnancy rate 86.2% ± 5—mean implantation sites 10.5 ± 0.4; mice, pregnancy rate 76.3% ± 4—mean implantation sites 7.6 ± 0.6; guinea-pigs, pregnancy rate 67.7% ± 7—mean implantation sites 2.2 ± 0.2.

*Chronic treatment.* Table 3 shows that a significant increase in the enzymatic activity for all the substrates used appears in each group of rats after 30-day treatment with the three combinations when the animals were killed 18 h after the last SCD administration. A dose-effect relationship is present for the combination of lynestrenol plus mestranol which was tested at three different doses. The treatments corresponding to groups II, V and VI produced a comparable decrease of fertility in rats.

*Effects of treatment 2 h before the animals were killed.* Results obtained in rats killed 2 h after the last administration at the end of 4 or 30 day treatment with the highest dose of the SCD combinations showed that such treatments did not modify the activity of the liver microsomal enzymes in comparison with the activity of these enzymes in control rats. These results, which are not given here in detail, indicate that, at least in rats, the inductive effect on the liver microsomal enzyme activity produced by these contraceptive agents does not appear during the first 2 h after the treatment.

### *Effects of treatment with contraceptive drugs on liver microsomal enzyme activity in mice*

*Acute treatment.* The effects of acute treatment (4 days) corresponding to one oestrous cycle with several doses of the three SCDs, was studied in mice. The data in Table 4 show that lynestrenol plus mestranol and norethisterone plus mestranol are very active

**Table 2** Effects of acute treatment with contraceptive drugs on liver microsomal activity in rats

| *Treatment (mg/kg per day, orally for 4 days)* | | *Enzymatic activity (nmol g$^{-1}$ h$^{-1}$ ± s.e.)* | | |
|---|---|---|---|---|
| | | *Aminopyrine* | *Aniline* | *pNO$_2$Anisole* |
| Controls | | 168±15 | 683±36 | 532±25 |
| Lynestrenol<br>+ mestranol | (5)<br>(0.3) | 189±13 | 606±33 | 498±27 |
| Norethisterone<br>+ mestranol | (4)<br>(0.2) | 326±20* | 787±31* | 695±24* |

Each figure represents the mean value for 8 animals. Rats were killed 18 h after the 4th day of treatment. Enzymatic activity is represented by the metabolites formed, 4-aminoantipyrine, *p*-NH$_2$ phenol, and *p*-NO$_2$-phenol, respectively from the 9000 *g* supernatant fraction, corresponding to 640 mg of fresh liver.
* $P < 0.01$ versus controls.

**Table 3** Effects of chronic treatment with contraceptive drugs at various dose levels on liver microsomal enzyme activity in rats

| *Group* | *No. of animals* | *Treatment (mg/kg per day) orally for 30 days)* | | *Enzymatic activity (nmol g$^{-1}$ h$^{-1}$ ± s.e.)* | | |
|---|---|---|---|---|---|---|
| | | | | *Aminopyrine* | *Aniline* | *pNO$_2$Anisole* |
| I | 10 | Controls | | 230±25 | 667±68 | 615±46 |
| II | 10 | Lynestrenol<br>+ mestranol | (1.25)<br>(0.075) | 379±29* | 829±57 | 803±34* |
| III | 10 | Lynestrenol<br>+ mestranol | (2.5)<br>(0.15) | 485±33* | 977±35* | 876±34* |
| IV | 10 | Lynestrenol<br>+ mestranol | (5)<br>(0.30) | 530±24* | 1100±41* | 892±28* |
| V | 7 | Norethisterone<br>+ mestranol | (2)<br>(0.1) | 435±36* | 1233±36* | 794±38* |
| VI | 7 | Norethynodrel<br>+ mestranol | (2)<br>(0.03) | 324±20* | 1081±49* | 738±27* |

Animals were killed 18 h after 30th day of treatment. The experimental conditions were as described in Table 2.
* $P < 0.01$ versus controls.

**Table 4** Effect of acute treatment with contraceptive drugs on liver microsomal enzyme activity in mice

I1100

| *Treatment (mg/kg orally) daily × 4 days* | | *Enzymatic activity (nmol $g^{-1}$ $h^{-1}$ ± s.e.)* *Aminopyrine* | *Aniline* | *p$NO_2$Anisole* |
|---|---|---|---|---|
| Controls | | 392 ± 21 | 626 ± 22 | 821 ± 16 |
| Lynestrenol<br>+ mestranol | (5)<br>(0.3) | 707 ± 27* | 1034 ± 112* | 1337 ± 34* |
| Lynestrenol<br>+ mestranol | (1.25)<br>(0.075) | 609 ± 18* | 885 ± 67* | 1181 ± 4* |
| Norethisterone<br>+ mestranol | (4)<br>(0.2) | 686 ± 34* | 828 ± 33* | 1069 ± 58* |
| Norethisterone<br>+ mestranol | (2)<br>(0.1) | 608 ± 39* | 863 ± 22* | 1092 ± 30* |
| Norethisterone<br>+ mestranol | (1)<br>(0.05) | 473 ± 21** | 709 ± 21** | 922 ± 20** |
| Norethynodrel<br>+ mestranol | (4)<br>(0.06) | 335 ± 9 | 511 ± 23 | 721 ± 42 |
| Norethynodrel<br>+ mestranol | (2)<br>(0.03) | 432 ± 25 | 672 ± 28 | 812 ± 29 |
| Norethynodrel<br>+ mestranol | (1)<br>(0.015) | 389 ± 57 | 567 ± 66 | 713 ± 75 |

Each figure is the mean of 5 determinations. The animals were killed 18 h after the 4th administration. Experimental conditions are described in Table 2.

* $P < 0.01$ versus controls; ** $P < 0.05$ versus controls.

**Table 5** Effect of mestranol and lynestrenol on microsomal enzyme activity in rats and mice

| *Animal species* | *Treatment (mg/kg per day, orally)* | | *No. of days* | *Enzymatic activity (nmol $g^{-1}$ $h^{-1}$ ± s.e.)* *Aminopyrine* | *Aniline* | *p$NO_2$Anisole* |
|---|---|---|---|---|---|---|
| Rat | Controls | | | 243 ± 11 | 790 ± 62 | 501 ± 40 |
| | Mestranol | (0.3) | 30 | 282 ± 20 | 837 ± 48 | 691 ± 51* |
| | Lynestrenol | (5) | 30 | 345 ± 23* | 1135 ± 126* | 819 ± 36* |
| | Lynestrenol<br>+ mestranol | (5)<br>(0.3) | 30 | 530 ± 24* | 1100 ± 41* | 892 ± 28* |
| Mouse | Controls | | | 416 ± 20 | 916 ± 34 | 1039 ± 40 |
| | Mestranol | (0.3) | 4 | 543 ± 14* | 774 ± 89 | 1033 ± 35 |
| | Lynestrenol | (5) | 4 | 593 ± 18* | 916 ± 69** | 1303 ± 86** |
| | Lynestrenol<br>+ mestranol | (5)<br>(0.3) | 4 | 707 ± 27* | 1034 ± 112* | 1337 ± 34* |

Each figure is the mean from at least 6 animals. Animals were killed 18 h after the last treatment. Experimental conditions as described in Table 2.

* $P < 0.01$ versus controls; ** $P < 0.05$ versus controls.

**Table 6** Effect of contraceptive drugs on liver microsomal proteins and cytochrome P 450

| Animal species | No. of animals per group | Treatment (mg/kg, orally) | | No. of days | Final body wt. (g ± s.e.) | Body wt. change (g ± s.e.) | Liver wt. (g/100 g body wt.) | Microsomal protein (mg/g ± s.e.) | (Cytochrome P 450 (nmol/mg protein) |
|---|---|---|---|---|---|---|---|---|---|
| Rat | 10 | Controls | | | 244 ± 5 | +41 ± 5 | 4.3 ± 0.02 | 19 ± 0.6 | 0.410 ± 0.03 |
| | 10 | Lynestrenol<br>+ mestranol | (1.25)<br>(0.075) | 30 | 221 ± 2 | +36 ± 2 | 4.4 ± 0.17 | 22 ± 1.4 | 0.424 ± 0.04 |
| | 10 | Lynestrenol<br>+ mestranol | (2.5)<br>(0.15) | 30 | 225 ± 2 | +34 ± 5 | 4.5 ± 0.13 | 21 ± 1.2 | 0.410 ± 0.04 |
| | 5 | Lynestrenol<br>+ mestranol | (5)<br>(0.3) | 30 | 204 ± 3 | +17 ± 3* | 4.8 ± 0.19 | 24 ± 2.7 | 0.484 ± 0.04 |
| Mouse | 5 | Controls | | 4 | 25.5 ± 0.4 | +0.6 | 5.5 ± 0.14 | 16 ± 0.5 | 0.305 ± 0.03 |
| | 5 | Lynestrenol<br>+ mestranol | (5)<br>(0.3) | 4 | 28.3 ± 0.7 | +1.1 | 5.9 ± 0.20 | 15 ± 0.4 | 0.390 ± 0.03 |

Body weight was measured before the first treatment and immediately before animals were killed, 18 h after the last treatment.
* $P < 0.01$ versus controls

inducers of microsomal enzymes even at the lowest doses tested. This effect appears in mice as in rats, at doses that do not exert a complete antifertility action.

On the other hand, combined treatment with norethynodrel and mestranol did not affect microsomal activity in mice. This SCD combination was also inactive at effective antifertility doses when given for a period of 30 days.

### *Comparison between the effects of oestrogen and progestogenic compounds on microsomal enzyme activity in rats and mice*

The following experiment was performed to test whether the inducing effect exerted by the contraceptive combination is dependent on the oestrogen or progestogenic compounds or on both. Rats and mice were treated for 30 and 4 days respectively, with lynestrenol and mestranol at the highest dose (5 and 0.3 mg/kg orally) used in the combined treatment in the previous experiments. The results are given in Table 5 and show that a significant increase in drug metabolism *in vitro* was always obtained when lynestrenol was present in the treatment. The effect of the mestranol is lacking or at the border line of significance. The data reported here suggest that the induction produced by the drug combination of lynestrenol plus mestranol is probably dependent only on the activity of the progestinic compound, lynestrenol as far as the aromatic hydroxylation and *O*-demethylation reactions are concerned. For the aminopyrine demethylation a synergistic effect of the two steroids cannot be excluded.

### *Effects of SCDs on liver weight, microsomal proteins and cytochrome P 450 concentration in rats and mice*

After combined treatments with lynestrenol and mestranol at doses capable of producing a significant induction in the liver microsomal enzyme activity of rats and mice, neither liver enlargement, nor an increase in liver micosomal proteins and cytochrome P 450 concentration were observed. The only significant observation was a reduction in body weight gain in the rats treated daily for 30 days with the highest dose of the combined treatment (Table 6).

### *Effects of chronic treatment with SCDs on liver microsomal enzyme activity in guinea-pigs*

The effect of the contraceptive drugs on liver microsomal enzyme activity in guinea-pigs was studied after a 32 day treatment, a period which corresponds to two oestrous cycles in this animal species. The results shown in Table 7 indicate that lynestrenol plus mestranol and norethisterone plus mestranol treatment at doses that are effective in reducing fertility in this species did not significantly modify the activity of the liver microsomal enzymes. However, the guinea-pigs were sensitive to the inducing activity of other drugs known to stimulate microsomal drug metabolism, such as phenobarbitone and eucalyptol.

**Table 7** Effect of combined treatment with contraceptive drugs on liver microsomal enzyme activity in guinea-pigs

| No. of animals | Treatment (mg/kg) and route | | No. of days | Enzymatic activity (nmol $g^{-1}$ $h^{+1}$ ± s.e.) | | |
|---|---|---|---|---|---|---|
| | | | | Aminopyrine | Aniline | p$NO_2$Anisole |
| 10 | Controls | | | 302 ± 28 | 767 ± 37 | 1592 ± 132 |
| 5 | Lynestrenol<br>+ mestranol | (0.3) oral<br>(0.018) | 32 | 374 ± 47 | 843 ± 35 | 1704 ± 67 |
| 10 | Lynestrenol<br>+ mestranol | (1.25) oral<br>(0.075) | 32 | 311 ± 27 | 627 ± 23 | 1596 ± 88 |
| 5 | Lynestrenol<br>+ mestranol | (5) oral<br>(0.3) | 32 | 304 ± 19 | 565 ± 14 | 1599 ± 17 |
| 5 | Norethisterone<br>+ mestranol | (4) oral<br>(0.2) | 32 | 331 ± 18 | 812 ± 49 | 1511 ± 82 |
| 5 | Phenobarbitone | (80) i.p. | 2 | 985 ± 153* | 1195 ± 76* | 2836 ± 281* |
| 5 | Eucalyptol | (500) s.c. | 3 | 769 ± 44* | 847 ± 43 | 2188 ± 104* |

The activity was measured on the supernatant of the 9000*g* fraction of the liver homogenate corresponding to 320 mg of fresh tissue. Experimental conditions as described in Table 2.

* $P < 0.01$ versus controls.

## Discussion

The results described here, confirm our previous data (Jori *et al.*, 1969) and indicate that the three SCD treatments tested enhance, although to various extents, the activity of the liver microsomal enzymes in rats and mice. This effect is probably produced by the progestogenic compounds alone, because, at least for one of the combined treatments tested in two animal species, the oestrogen alone is almost devoid of activity. These results agree with those previously obtained by our laboratory (Jori *et al.*, 1969) and by other authors (Blackham & Spencer, 1969), concerning the effect of oestrogens on drug metabolism *in vivo* and *in vitro*. However a potentiating effect of the oestrogens on the activity of the progestogens cannot be excluded at least for some substrates.

We were unable to detect any decrease in the activity of the liver microsomal enzymes after combined contraceptive treatments in mice and rats. The inhibition of drug metabolism by the progestogens was generally found in experiments *in vitro* and at very high concentrations (Soyka & Long, 1972; Tüttenberg *et al.*, 1974). *In vivo* only norethynodrel has been reported to inhibit pentobarbitone metabolism (Juchau & Fouts, 1966; Jori *et al.*, 1969), after a single acute treatment. All these situations are quite different from the conditions of the *in vivo* chronic treatment used in our experiments.

The increase in liver microsomal enzyme activity after contraceptive drug treatment in mice and rats, is not associated with an increase of the microsomal proteins or of the cytochrome P 450 content, parameters generally indicative of enzyme induction. These results are not surprising because other steroids such as methyltestosterone, cortisone and spironolactone stimulate drug metabolism in female rats without increasing the cytochrome P 450 content (Hamrick, Zampaglione, Stripp & Gillette, 1973). Moreover these authors stated that the rate of drug metabolism is determined by factors which cannot be directly related to cytochrome P 450 content, cytochrome reductase activity and magnitude of spectral changes.

Our data are at variance with the findings of Freudenthal & Amerson (1974) who showed that oestrogen–progestogen combinations are not microsomal enzyme inducers. Possible explanations for these differences are the following: first their conclusions are based mainly on the lack of an increase in P 450 content and in other components of the microsomal electron transport system, after progestogenic treatment; second, they used male rats and it has been reported that other steroids increase microsomal metabolism only in female rats (Stripp, Hamrick & Zampaglione, 1970; Hamrick *et al.*, 1973); finally, in their experiments the chronic treatment was repeated for only 4 days. In our experiments contraceptive treatment was always active in mice after a few administrations whereas rats, at least for lynestrenol plus mestranol combination, needed a chronic treatment of more than 4 days to show a significant inducing effect.

However, our aim was to study the activity of liver microsomal enzymes, in experimental conditions reproducing as closely as possible, the pattern of use of the contraceptives in human therapy and consequently for periods covering more than one oestrous cycle. An objection could be raised that the modifications in microsomal enzyme activity produced by the various treatments are present only at very high doses as compared with those used in human contraceptive treatment; but it must be noted that in fact, the doses used are very low in relation to the antifertility effect exerted by such treatment in rats and mice. A significant increase in enzymatic activity appears at doses unable to protect all the animals from pregnancy.

The reactivity of the guinea-pigs to the contraceptive combined treatments is quite different from that of mice and rats. This animal species is very sensitive to the antifertility action of the contraceptive drugs, but is is unresponsive to their microsomal enzyme-inducing activity. It is difficult to explain this lack of response on the basis of a poor availability or a different kinetic behaviour of the progestogenic compounds in this animal species because they exert at these doses a high antifertility action, although a more specific distribution of the steroids in the target reproductive organs compared with the liver cannot be excluded in this species.

However, it may be more likely that this different reactivity of the guinea-pig is due to the widely recognized species differences in microsomal drug metabolism (Parke, 1968). Differences between mice and guinea-pigs in biochemical composition and enzyme distribution in microsomal sub fractions have been reported recently (Gram, Schroeder, Davis, Reagen & Guarino, 1971).

We are indebted to Dr A. Bonaccorsi and Dr R. Franco for the data on the antifertility activity of the contraceptive drugs obtained by their laboratory. This study was supported by NIH contract No. NIH-NICHD-72-2733.

**References**

BLACKHAM, A. & SPENCER, P.S.J. (1969). The effects of oestrogens and progestins on the response of mice to barbiturates. *Br. J. Pharmac.*, **37**, 129–139.

CONNEY, A.H. (1967). Pharmacological implications of microsomal enzyme induction. *Pharmac. Rev.*, **19**, 317–366.

CRAWFORD, J.S. & RUDOFSKY, S. (1966). Some alterations in the pattern of drug metabolism associated with pregnancy, oral contraceptives, and the newly-born. *Br. J. Anaesth.*, **38**, 446–454.

FREUDENTHAL, R.I. & AMERSON, E. (1974). Effect of synthetic estrogens and estrogen–progestin combinations on the hepatic microsomal enzyme system. *Biochem. Pharmac.*, **23**, 2651–2656.

GARG, R.C. & AHMAD, A. (1974). Effect of some oral contraceptives on the response of mice to pentobarbitone. *Pharmac. Res. Comm.*, **6**, 47–54.

GILBERT, D. & GOLBERG, L. (1965). Liver response tests. 3. Liver enlargement and stimulation of microsomal processing enzyme activity. *Fd. Cosmet. Toxicol.*, **3**, 417–432.

GRAM, T.E., SCHROEDER, D.H., DAVIS, D.C., REAGAN, R.L. & GUARINO, A.M. (1971). Enzymic and biochemical composition of smooth and rough microsomal membranes from monkey, guinea pig and mouse liver. *Biochem. Pharmac.*, **20**, 1371–1381.

HAMRICK, M.E., ZAMPAGLIONE, N.G., STRIPP, B. & GILLETTE, J.R. (1973). Investigation of the effects of methyltestosterone, cortisone and spironolactone on the hepatic microsomal mixed function oxidase system in male and female rats. *Biochem. Pharmac.*, **22**, 293–310.

JORI, A., BIANCHETTI, A. & PRESTINI, P.E. (1969). Effect of contraceptive agents on drug metabolism. *Eur. J. Pharmac.*, **7**, 196–200.

JUCHAU, M.R. & FOUTS, J.R. (1966). Effects of norethynodrel and progesterone on hepatic microsomal drug-metabolizing enzyme systems. *Biochem. Pharmac.*, **15**, 891–898.

KATO, R. & TAKANAKA, A. (1967). Effect of starvation on the "in vivo" metabolism and effect of drugs in female and male rats. *Jap. J. Pharmac.*, **17**, 208–217.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

O'MALLEY, K., STEVENSON, I.H. & CROOKS, J. (1972). Impairment of human drug metabolism by oral contraceptive steroids. *Clin. Pharmac. Ther.*, **13**, 552–557.

OMURA, T. & SATO, R. (1964). The carbon monoxide-binding pigment of liver microsomes. *J. biol. Chem.*, **239**, 2370–2378.

PARKE, D.V. (1968). *Biochemistry of Foreign Compounds*, p. 113. Oxford: Pergamon Press.

RÜMKE, Chr. L. & NOORDHOEK, J. (1969). The influence of lynestrenol on the rate of metabolism of phenobarbital, phenytoin and hexobarbital in mice. *Eur. J. Pharmac.*, **6**, 163–168.

SCHENKMAN, J.B. & CINTI, D.L. (1972). Hepatic mixed function oxidase activity in rapidly prepared microsomes. *Life Sci.*, **11**, 247–257.

SOYKA, L.F. & DECKERT, F.W. (1974). Further studies on the inhibition of drug metabolism by pregnanolone and related steroids. *Biochem. Pharmac.*, **23**, 1629–1639.

SOYKA, L.F. & LONG, R.J. (1972). "In vitro" inhibition of drug metabolism by metabolites of progesterone. *J. Pharmac. exp. Ther.*, **182**, 320–327.

STRIPP, B., HAMRICK, M. & ZAMPAGLIONE, N. (1970). Effect of spironolactone treatment of rats on the oxidation of drugs by liver microsomes. *Fedn Proc.*, **29**, 346.

TEPHLY, T.R. & MANNERING, G.J. (1968). Inhibition of drug metabolism. V. Inhibition of drug metabolism by steroids. *Molec. Pharmac.*, **4**, 10–14.

TÜTTENBERG, HÜTHWOHL, B., KAHL, R. & KAHL, G.F. (1974). Effects of synthetic progestagens on drug metabolism in rat liver microsomes. *Biochem. Pharmac.*, **23**, 2037–2043.

WHITTEN, W.K. (1957). Effect of exteroceptive factors on the oestrous cycle of mice. *Nature, Lond.*, **180**, 1436.

WHITTEN, W.K. (1958). Modification of oestrous cycle of the mouse by external stimuli associated with the male. *J. Endocr.*, **17**, 307–313.

*(Received September 29, 1975.*
*Revised April 26, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 183–188

# MYOCARDIAL DIGOXIN UPTAKE: DISSOCIATION BETWEEN DIGITALIS-INDUCED INOTROPISM AND MYOCARDIAL LOSS OF POTASSIUM

EVA STEINESS[1] & N. VALENTIN
Department of Pharmacology, Department of Medicine B and Department of Thoracic Surgery R,
Division of Cardiac Surgery, Rigshospitalet, University of Copenhagen, Denmark

**1** The time course of myocardial uptake of digoxin, of increase in inotropic effect and of changes in myocardial potassium content were studied following a single intravenous dose of digoxin.

**2** Nineteen dogs with intact circulation were investigated by the use of a biopsy technique which allowed samplings before and 10, 30, 60, and 90 min after administration of digoxin.

**3** The myocardial concentration of digoxin was $196 \times 10^{-9}$ mol/kg 10 min after administration of digoxin. Uptake continued at a slower rate, maximum concentration being $293 \times 10^{-9}$ mol/kg at 60 minutes.

**4** The inotropic effect increased parallel with the uptake of digoxin; 10 min after digoxin, contractility was 127% of the control value and this increased to 139% at 90 minutes.

**5** Myocardial potassium content was slightly increased 10 min after digoxin, suggesting an initial stimulation of membrane $Na^+$-$K^+$ ATPase. A subsequent significant fall in the myocardial potassium content probably reflects ATPase inhibition.

**6** The temporal dissociation between the early onset of the positive inotropic effect and the delayed inhibition of membrane $Na^+$-$K^+$ ATPase indicates that inotropism of digitalis glycosides is not mediated by the same binding site as that responsible for inhibition of $Na^+$-$K^+$ ATPase.

## Introduction

It is well established that digitalis glycosides increase the force of myocardial contractility but the mechanism of this action is still unknown.

In 1953 Hajdu advanced the hypothesis that digitalis-induced inotropism is related to an inhibition of membrane $Na^+$-$K^+$ ATPase. Evidence supporting this concept was recently reviewed by Langer (1972). He concluded that the positive inotropic effect of digitalis is not seen, unless inhibition of the ATPase enzyme is also observed. However, his arguments were based on studies performed with relatively high concentrations of digitalis either on isolated, perfused heart preparations or on isolated myocardial muscle preparations. Studies of the isolated $Na^+$-$K^+$ ATPase enzyme have revealed that low concentrations of digitalis stimulate enzyme activity while higher concentrations inhibit it (Lee & Yu, 1963; Palmer & Nechay, 1964; Oppelt & Palmer, 1966). Stimulation of $Na^+$-$K^+$ ATPase increases intracellular potassium while enzyme inhibition leads to reduction in potassium content. This biphasic interaction between digitalis and $Na^+$-$K^+$ ATPase and its relation to myocardial performance and uptake of digoxin have not been studied in intact animals.

The present study, using a biopsy technique allowing serial samplings from the same myocardium was undertaken in order to follow the temporal uptake of digoxin by the intact canine heart and to correlate this with the inotropic effect and changes in myocardial potassium.

## Methods

Nineteen dogs weighing 18.5–25 kg were divided into three groups. Group 1: Eight dogs were given digoxin and serial myocardial biopsies were taken. Group 2: Seven dogs were given digoxin but biopsies were taken only at the end of the study. The group served as a control for haemodynamic parameters. Group 3: Four dogs received no digoxin and served as a control for the entire experimental procedure.

### *Experimental procedure*

Anaesthesia was induced with 300–500 mg of narcobarbital (Narcodorm). After relaxation with tubocurarine, orotracheal intubation was performed and the animal was connected to a respirator (Bird Mark 10) via a ventimeter (Airshield V-2) to ensure constant ventilation. Anaesthesia was maintained with $N_2O/O_2$ (flow 3 and 1.5 l/min, respectively) in a to-and-fro system. The anaesthetic together with pethidine (10 mg/kg), given once or twice an hour when needed, abolished pain reflexes and only when absence of pain had been assured was (+)-tubocurarine (10 mg i.v.) given to relax the chest and so facilitate the operative procedure.

---

[1] Present address: Department of Pharmacology, University of Copenhagen, Juliane Maries Vej 20, DK-2100 Copenhagen, Denmark.

The heart was exposed through a left-sided anterior thoracotomy. The left atrium was catheterized through the auricle for pressure recording and blood sampling. Retrograde catheterization of the left ventricle was performed with a catheter of the pig tail type. Atrial and ventricular pressures were measured by the use of Elema Schönander transducers (type EMT 35) (resonant frequency ($F_D$) of the catheter transducer system = 20 Hz and damping factor ($\beta$) = 0.2). The first derivative of the ventricular pressure curve, was obtained by means of a differentiating unit with a cut-off frequency of 338 Hz (King & Searle, 1972). Since completing these studies, similar results were obtained using a catheter with an $F_D$ of about 35 Hz, $\beta$ of 0.7 and a cut off frequency of the differentiating unit of about 600 Hz (N.E. Bille-Brahe, E. Steiness & N. Lomholt, unpublished observations).

The haemodynamic parameters and a standard limb lead of the ECG were displayed simultaneously on an Elema Schönander multichannel recorder (Mingograf 81).

When haemodynamic stability was reestablished (usually after 10–15 min), pethidine and tubocurarine were discontinued and halothane was added to the anaesthetic mixture in a concentration (0.5–0.7%) sufficient to cause 20–50% depression of the initial $dp/dt_{max}$; 60 min were allowed for stabilization at this new level before digoxin, 0.05 mg/kg body weight, was given as an intravenous bolus injection.

### *Blood samples*

Heparinized arterial blood samples were drawn before and 5, 10, 15, 20, 30, 45, 60, 75, and 90 min after the injection of digoxin for measurements of plasma concentrations of digoxin, sodium, and potassium.

Arterial $P_{O_2}$, $P_{CO_2}$, and pH were determined before and 10, 30, 60, and 90 min after the administration of digoxin.

### *Biopsies*

A knife biopsy weighing 200–400 mg was taken from the left ventricular wall 15 min before the administration of digoxin in all dogs. Great care was taken to avoid cutting coronary vessels. The myocardial incision was closed with a continuous silk suture. In group 1 and 3, myocardial biopsies were again taken at 10, 30, 60, and 90 minutes. In group 2, a biopsy was taken again at 90 min only.

Any blood loss, including sampling, was replaced with an electrolyte-containing plasma expander (Haemaccel), and the estimated loss of water by evaporation was replaced with isotonic glucose.

All the animals were finally killed with intravenous narcobarbital.

### *Analytical procedures*

Plasma digoxin concentrations were measured in duplicate by radioimmunoassay (Steiness, 1974).

Plasma potassium and sodium were determined in duplicate by flame photometry (Instrumentation Laboratory 143). Blood gases and pH were measured with a Radiometer.

The myocardial biopsies were divided into four pieces, one of which was used for determination of digoxin and the others for measurement of potassium.

The tissue content of digoxin was estimated by the cited radioimmunological method, following homogenization in 0.9% w/v NaCl solution (saline) and extraction with dichloromethane (Steiness, 1974). The sensitivity of the method used was $10 \times 10^{-9}$ mol/kg tissue. The recovery of known amounts of digoxin added to the myocardial homogenates averaged 95% (s.d. = ±6%) between 50 and 1000 mol × $10^{-9}$/kg tissue.

Tissue content of potassium was determined in triplicate with a s.d. of 19 mmol/kg fat-free tissue according to Valentin & Olesen (1973). Briefly, the specimens were weighed before and after drying and after extraction of fat to determine the content of water and fat-free solids. The tissue content of potassium was measured following extraction with nitric acid and expressed in mmol/kg fat-free solids.

### *Statistics*

The Wilcoxon test for paired data was used for the determination of statistical significance.

## Results

### *Digoxin*

After injection, similar plasma digoxin curves were obtained in groups 1 and 2 (Figure 1). Plasma concentrations continued to fall throughout the study.

Left ventricular digoxin concentration increased after intravenous injection and reached a median value of 67% of the maximum median value within 10 min (Table 1). A significant uptake continued beyond the first 10 min, since the median value was significantly higher after 60 min than after 10 min ($P < 0.02$,

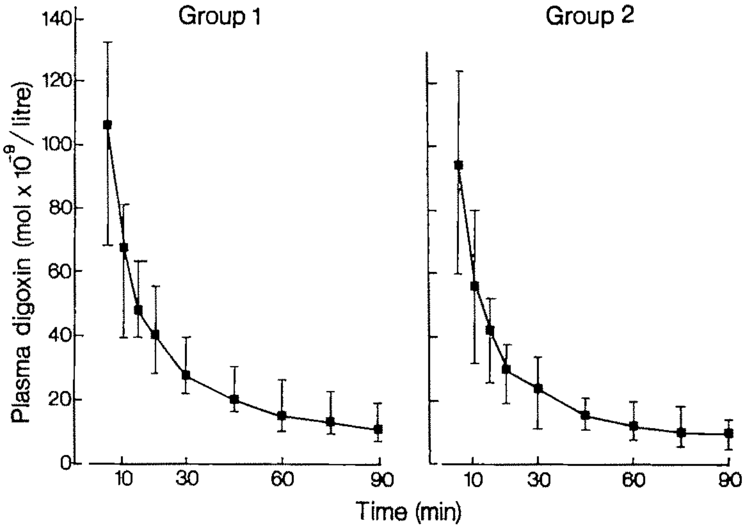


**Figure 1** Plasma digoxin concentrations (median) in dogs following an intravenous dose of 0.05 mg digoxin/kg body weight. The vertical lines represent the ranges.

group 1). The peak myocardial digoxin concentration was reached between 30 and 90 min (Figure 2). No significant difference between groups 1 and 2 was found in the myocardial content of digoxin at 90 minutes.

*Electrolytes*

Only minor changes in plasma potassium were observed and plasma concentration of sodium remained unchanged throughout the study. The

**Table 1** Myocardial digoxin concentration (mol x $10^{-9}$/kg tissue and in parentheses μg/kg tissue) in dogs following an intravenous injection of 0.05 mg digoxin/kg body weight

| | | Minutes after digoxin | | | | |
|---|---|---|---|---|---|---|
| *Group* | | *0* | *10* | *30* | *60* | *90* |
| 1 | Median | 0 | 196 (154) | 228 (179) | 293 (230) | 237 (186) |
| *n*=8 | Range | | 166–284 | 189–376 | 136–385 | 150–467 |
| 2 | Median | — | — | — | — | 226 (177) |
| *n*=7 | Range | | | | | 188–406 |

**Table 2** Myocardial potassium content (mmol/kg fat-free solids) in dogs following intravenous administration of 0.05 mg digoxin/kg body weight

| | | Minutes after digoxin | | | | |
|---|---|---|---|---|---|---|
| *Group* | | *0* | *10* | *30* | *60* | *90* |
| 1 | Median | 375 | 387 | 365 | 353 | 358* |
| *n*=8 | Range | 340–462 | 350–475 | 273–431 | 330–427 | 334–402 |
| 2 | Median | 383 | — | — | — | 357* |
| *n*=7 | Range | 351–429 | | | | 321–408 |
| 3 | Median | 318 | 300 | 307 | 327 | 321 |
| *n*=4 | Range | 250–341 | 277–300 | 266–396 | 266–361 | 266–394 |

* The pooled values for groups 1 and 2 were significantly lower at 90 min than at 0 min ($P<0.01$).

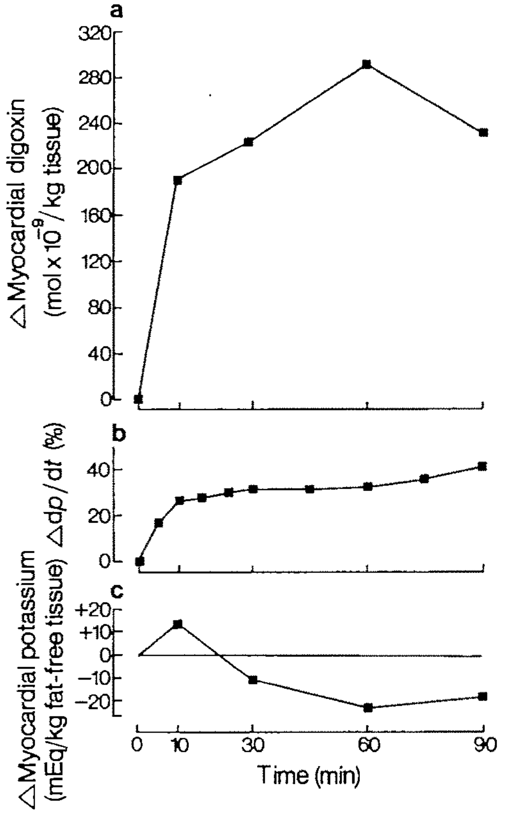


**Figure 2** Median values of (a) myocardial digoxin concentration, (b) left ventricular contractility ($dp/dt_{max}$), and (c) myocardial potassium in dogs following an intravenous injection of digoxin (0.05 mg/kg body weight). The $dp/dt$ values are from group 2 (no cardiac surgery between 0 and 90 min, the other data from group 1.

anaesthetic and experimental procedures themselves caused no change of the plasma concentrations of potassium and sodium.

Following a slight initial increase (insignificant) the median myocardial potassium concentrations decreased during the study period (Table 2). Myocardial potassium concentrations were similar in groups 1 and 2 before administration of digoxin and both groups showed a similar reduction at 90 minutes. By contrast the experimental procedure itself caused no change in the myocardial potassium, although the zero level of myocardial potassium was, for unknown reasons, significantly lower in group 3 than in groups 1 and 2.

*Haemodynamic parameters*

As shown in Table 3, left ventricular $dp/dt_{max}$ increased during the first 10 min in both groups 1 and

**Table 3** Left ventricular contractility ($dp/dt_{max}$) following intravenous administration of 0.05 mg digoxin/kg body weight

| *Group* | | *Minutes after digoxin* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *0* | *5* | *10* | *15* | *20* | *30* | *45* | *60* | *75* | *90* |
| 1 | Median | 100 | 119 | 121 | 112 | 114 | 122 | 119 | 124 | 122 | 118 |
| *n* = 8 | Range | — | 105–150 | 110–135 | 100–130 | 100–136 | 105–155 | 89–143 | 95–150 | 84–154 | 89–147 |
| 2 | Median | 100 | 116 | 127 | 127 | 127 | 129 | 131 | 137 | 137 | 139 |
| *n* = 7 | Range | — | 100–142 | 104–167 | 104–167 | 108–150 | 108–163 | 113–175 | 113–208 | 113–192 | 100–200 |
| 3 | Median | 100 | 100 | 96 | 105 | 100 | 100 | 95 | 100 | 100 | 105 |
| *n* = 4 | Range | — | 90–107 | 90–110 | 96–107 | 90–110 | 96–105 | 90–105 | 95–107 | 95–106 | 90–110 |

2. In group 1 $dp/dt_{max}$ tended to fall after each biopsy and the slight increase at 90 min was insignificant. In group 2 there was a similar rapid increase in contractility (Table 3), but in these animals in contrast to those of group 1, peak $dp/dt_{max}$ remained stable after 10 minutes.

Other haemodynamic parameters, left ventricular systolic and end-diastolic pressures, left atrial mean pressure and heart rate remained unchanged during the study.

### *Blood gases*

Arterial $P_{O_2}$, $P_{CO_2}$, and pH did not change significantly during the study.

## Discussion

Previous studies of myocardial digoxin uptake have been performed either on isolated muscles (Prindle, Skelton, Epstein & Marcus, 1971) or using intact animals, which were killed at different times after the administration of digoxin (Cohn, Kleiger & Harrison, 1967; Deutscher, Harrison & Goldman, 1972; Goldman, Deutscher, Schweizer & Harrison, 1972; Hopkins, Lloyd & Taylor, 1974). Most measurements were performed at 30 to 60 min after the administration of digoxin, when a myocardial digoxin of about 200–300 mol × $10^{-9}$/kg tissue was found.

Deutscher *et al.* (1972), Goldman *et al.* (1972) and Hopkins *et al.* (1974) studied myocardial uptake as early as 5 to 15 min after administration of digoxin in a small series of intact dogs and found, even at these times, a high myocardial content of digoxin. However, individual variations in the digoxin content were large. These methods require one animal for each determination and are poorly suited for study of the temporal pattern of uptake.

With the sequential biopsy technique used in this study it was shown that the myocardial digoxin concentration was high 10 min after injection, confirming the results of Deutscher *et al.* (1972), Goldman *et al.* (1972), and Hopkins *et al.* (1974). In addition a significant uptake of digoxin was shown to continue for at least 60 min after administration. A tendency towards a fall in myocardial digoxin content between 60 and 90 min perhaps reflects the early phase of elimination from tissues (Figure 2).

Immediately after intravenous digoxin, plasma concentrations were high. However, even at 5 min the plasma concentration was lower than the total myocardial content at 10 minutes. Plasma protein binding in dogs is less than in humans, being 15 and 25%, respectively (Steiness & Rasmussen, unpublished data), and the total extracellular space of the myocardial tissue amounts to approximately 30% tissue weight (Glitsch, 1972). Consequently, less than 10% of the myocardial digoxin content at 10 min was present in the extracellular myocardial fluid. In conformity with other papers dealing with this subject, the total myocardial digoxin content has, therefore, been given without correction. The present investigation, in agreement with studies of the subcellular fractions of myocardial digoxin (Conrad & Baxter, 1964; Dutta, Goswami, Lindower & Marks, 1968) suggests that the myocardial uptake takes place by rapid active cellular transport.

A significant positive inotropic effect was demonstrated 5 min after intravenous administration of digoxin to dogs with a controlled and stable anaesthetic cardiodepression. The increase of the positive inotropic effect continued from 5 to 10 min and in accordance with the results of Deutscher *et al.* (1972) and Goldman *et al.* (1972) we were unable to demonstrate further significant increases of the contractility after 15 minutes.

A transient decrease of $dp/dt_{max}$ was observed after a biopsy. The myocardial uptake of digoxin and the changes of myocardial potassium content (group 1) are therefore compared to the haemodynamic parameters from the series not subjected to repeated biopsy (group 2). The two groups are comparable since no significant difference was found between the plasma digoxin curves (Figure 1), the myocardial content of potassium before and 90 min after digoxin (Table 2), or the myocardial content of digoxin 90 min after digoxin (Table 1).

The development of the positive inotropic effect during the first 10 min seemed related to the myocardial uptake of digoxin. On the other hand, the myocardial digoxin content continued to increase up to 60 min without a corresponding increase of the positive inotropic effect (Figure 2). The digoxin uptake between 10 and 60 min may, therefore, not be correlated to further digoxin binding at the binding sites responsible for inotropic effect.

It is well established that digitalis glycosides inhibit the active $Na^+$-$K^+$ transport across the cell membrane, causing a decrease of the intracellular potassium content. In accordance with this well-described phenomenon, a loss of myocardial potassium was observed between 30 and 90 min after administration of digoxin, while no significant change was observed at 10 minutes. The results suggest a possibility of a change of the myocardial content of potassium following an intravenous digoxin dose. Such a possibility would accord with the results from studies of the effect of digitalis on the isolated $Na^+$-$K^+$ ATPase enzyme (Lee & Yu, 1963; Palmer & Nechay, 1964; Oppelt & Palmer, 1966). On the other hand, measurements of the myocardial cellular influx and efflux of potassium have revealed that whereas low doses of digoxin inhibit the potassium efflux more than the corresponding potassium influx and thereby increase the cellular potassium content; higher doses of digoxin inhibit the potassium influx more than the corresponding efflux and thereby reduce the cellular content of potassium (Grupp & Charles, 1964). The initial minor increase (non-significant) of the myocardial content of potassium observed in the present study could be due to either a greater initial inhibition of the potassium efflux than that of potassium influx, or an initial stimulation of the ATPase.

The positive inotropic effect was demonstrated shortly after intravenous administration of digoxin, and a near maximum effect was reached by 10 min, when the myocardial concentration of digoxin was high. At the same time, evidence of low ATPase digoxin binding was demonstrated. Subsequent binding of digoxin at the 'ATPase binding site' was not followed by a major increase of the positive inotropic effect.

These observations suggest that the positive inotropic effect is independent of the digoxin effect on the $Na^+$-$K^+$ ATPase. This is in agreement with the results from wash-out studies in isolated heart preparations perfused with a constant concentration of digitalis (Okita, Richardson & Roth-Schechter, 1973; Peters, Raben & Wassermann, 1974). In these studies the ATPase inhibition was washed out more rapidly than the positive inotropic effect. In accordance with these investigations the present results lead to the assumption that at least two different binding sites for digitalis are present in the myocardium. One of these is responsible for the inhibition of the membrane ATPase and the other for development of the positive inotropic effect.

This work was supported by grants from Statens Laegevidenskabelige Forskningsraad and Hjerteforeningen.

**References**

COHN, K.E., KLEIGER, R.E. & HARRISON, D.C. (1967). Influence of potassium depletion on myocardial concentration of tritiated digoxin. *Circulation Res.*, **20**, 473–476.

CONRAD, L.L. & BAXTER, D.J. (1964). Intracellular distribution of digoxin-$H^3$ in the hearts of rats and dogs demonstrated by autoradiography and its relationship to change in myocardial contractile force. *J. Pharmac. exp. Ther.*, **145**, 210–214.

DEUTSCHER, R.N., HARRISON, D.C. & GOLDMAN, R.H. (1972). The relation between myocardial $^3H$-digoxin concentration and its hemodynamic effects. *Am. J. Cardiol.*, **29**, 47–55.

DUTTA, S., GOSWAMI, S., LINDOWER, J.O. & MARKS, B.H. (1968). Subcellular distribution of digoxin-$H^3$ in isolated guinea-pig and rat hearts. *J. Pharmac. exp. Ther.*, **159**, 324–334.

GLITSCH, H.G. (1972). Hemmung der elektrogenen Na-Pumpe am Meerschweinschenvorhof durch Digitoxigenin. *Pflugers Arch.*, **335**, 243–251.

GOLDMAN, R.H., DEUTSCHER, R.N., SCHWEIZER, E. & HARRISON, D.C. (1972). Effect of a pharmacologic dose of digoxin on inotropy in hyper- and normokalemic dogs. *Am. J. Physiol.*, **223**, 1438–1443.

GRUPP, G. & CHARLES, A. (1964). Effect of ouabain and 3-acetyl strophantidin on potassium exchange in the dog heart in situ. *J. Pharmac. exp. Ther.*, **143**, 356–365.

HAJDU, S. (1953). Mechanism of staircase and contracture in ventricular muscle. *Am. J. Physiol.*, **174**, 371–380.

HOPKINS, B.E., LLOYD, B.L. & TAYLOR, R.R. (1974). Myocardial digoxin uptake in pressure overload right ventricular hypertrophy. *Cardiovasc. Res.*, **8**, 701–706.

KING, G.E. & SEARLE, J.P. (1972). A simple calibrator for measuring the first derivative of pressure waveforms. *IEEE Transactions on Biomedical Engineering (New York)*, **19**, 320–321.

LANGER, G.A. (1972). Effects of digitalis on myocardial ionic exchange. *Circulation*, **46**, 180–187.

LEE, K.S. & YU, D.H. (1963). A study of the sodium- and potassium-activated adenosinetriphosphatase activity of heart microsomal fraction. *Biochem. Pharmac.*, **12**, 1253–1264.

OKITA, G.T., RICHARDSON, F. & ROTH-SCHECHTER, B. (1973). Dissociation of the positive inotropic action of digitalis from inhibition of sodium- and potassium-activated adenosine triphosphatase. *J. Pharmac. exp. Ther.*, **185**, 1–11.

OPPELT, W.W. & PALMER, R.F. (1966). Stimulation of cerebrospinal fluid production by low doses of intraventricular ouabain. *J. Pharmac. exp. Ther.*, **154**, 581–585.

PALMER, R.F. & NECHAY, B.R. (1964). Biphasic renal effects of ouabain in the chicken: Correlation with a microsomal $Na^+$-$K^+$-stimulated ATPase. *J. Pharmac. exp. Ther.*, **146**, 92–98.

PETERS, T., RABEN, R.H. & WASSERMANN, O. (1974). Evidence for a dissociation between positive inotropic effect and inhibition of the $Na^+$-$K^+$-ATPase by ouabain, cassaine and their alkylating derivatives. *Eur. J. Pharmac.*, **26**, 166–174.

PRINDLE, JR., K.H., SKELTON, C.L., EPSTEIN, S.E. & MARCUS, F.I. (1971). Influence of extracellular potassium concentration on myocardial uptake and inotropic effect of tritiated digoxin. *Circulation Res.*, **28**, 337–345.

STEINESS, E. (1974). Renal tubular secretion of digoxin. *Circulation*, **50**, 103–107.

VALENTIN, N. & OLESEN, K.H. (1973). Measurement of muscle tissue water and electrolytes. *Scand. J. clin. Lab. Invest.*, **32**, 155–160.

*(Received October 6, 1975.*
*Revised April 7, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 189–192

# ON THE DECREASE OF MYOCARDIAL OXYGEN CONSUMPTION INDUCED BY CHLORACYZINE

I.E. KISIN

Moscow Medical Institute and School of Medicine in Haifa, P.O.B. 9649, Israel

**1** The effects of chloracyzine (2-chloro-10-(3-diethylaminopropionyl)-phenothiazine hydrochloride; antianginal drug) on myocardial oxygen consumption were studied in open-chest cats and cat isolated hearts using oximetry and polarography respectively.

**2** It was found that chloracyzine produced a decrease in myocardial oxygen consumption accompanied by a reduction in coronary blood flow preceded by transient coronary dilatation. Chloracyzine produced an insignificant increase in arterial pressure; heart rate increased slightly in the open-chest experiments but not in the isolated heart.

**3** It is suggested that reduced oxygen uptake after chloracyzine is realized through improved efficiency in the use of oxygen.

## Introduction

The use of $\beta$-adrenoceptor blocking agents for the treatment of angina pectoris emphasizes the importance of reduction of cardiac oxygen expenditure in relief of this disease. Decrease of compensatory sympathetic drive after propranolol and hence of heart work, often results in heart failure in patients who are on the border-line of it. Reduction of the myocardial demand for oxygen, without reducing heart activity was found in our studies of 2-chloro-10-(3-diethylaminopropionyl)-phenothiazine hydrochloride (chloracyzine), synthesized and investigated at the Moscow Institute of Pharmacology (Vikhljaev & Kaverina, 1959; Zhuravlev, Gritsenko & Ermakova, 1968). It was shown to have spasmolytic properties and prevent or relieve spasms of the coronary vessels, on which grounds it was proposed as an antianginal drug. Besides its effect on the coronary vessels, it exhibits an atropine-like activity, some ability to increase arterial pressor responses to catecholamines, and a moderate antihistaminic effect. It has been marketed in the USSR for many years, and used for the treatment of angina pectoris.

In the course of a systematic survey of the effect of different antianginal drugs on myocardial oxygen consumption, chloracyzine was found to have particularly interesting properties in this respect. This paper is concerned with its effect on the oxygen balance of the heart.

## Methods

### *Open-chest cat*

The experiments were performed on cats weighing 3–4 kg, anaesthetized with pentobarbitone (40 mg/kg, i.v.) and respired with a pump. The heart was exposed through a left thoracotomy, heparin (1,500 iu/kg) being used as anticoagulant. Arterial pressure was recorded from the carotid artery with a mercury manometer. Outflow from the coronary sinus was collected with a plastic cannula inserted into the sinus orifice via the right auricle. The cannula was held in a fixed position by a suture that was passed around the coronary sinus and tied. This arrangement excluded contamination of sinus blood with mixed venous blood. The position of the cannula was checked post mortem. The coronary venous outflow was recorded continuously with a Gaddum flowmeter. The blood was returned to the femoral vein by a pump. Coronary oxygen saturation was recorded continuously by directing the coronary outflow through a cuvette connected to a transmission-type oximeter. The arterial haemoglobin oxygen saturation was determined by taking arterial samples in the course of the experiment. The haemoglobin oxygen saturation of the blood samples was measured with a haemoreflector and haemoglobin content was determined by a photometer. Myocardial oxygen consumption was calculated by multiplying the coronary venous flow by the arterio-venous difference in haemoglobin oxygen saturation and oxygen capacity of the blood (haemoglobin content in mg/100 ml blood × 1.34). The effect of chloracyzine (2 or 5 mg/kg, i.v.) was compared with that of two other coronary antispasmodics, aminophylline (3 or 7 mg/kg, i.v.) and prenylamine (1 or 2 mg/kg, i.v.).

### *Isolated heart*

Under light ether anaesthesia the heart was quickly removed, suspended in a Langendorff apparatus and perfused via the aortic root with oxygenated Krebs solution (kept at 37°C) at a constant pressure of 80 cm water. The coronary perfusate of the beating heart was collected and measured with a Gaddum recorder. Oxygen content in the coronary outflow and inflow were recorded polarographically in the following manner. Krebs solution was continuously directed to a cell which held two electrodes. One of them was a platinum wire 0.2 mm in diameter, insulated by a glass covering throughout its length except for a distance of 1 mm at the tip. It formed the cathode for determination of oxygen content. The reference electrode was a Ag–AgCl plate (5 x 5 mm). A polarizing source, adjusted to 0.6 V, and a recording device were connected between the reference electrode and the cathode. The oxygen electrode was calibrated by Krebs solution with known oxygen content (for details see Kisin & Buyanov, 1963). Chloracyzine (0.2 or 0.5 mg) was injected into the aortic root through a catheter. Student's *t*-test was used for statistical analysis of results.

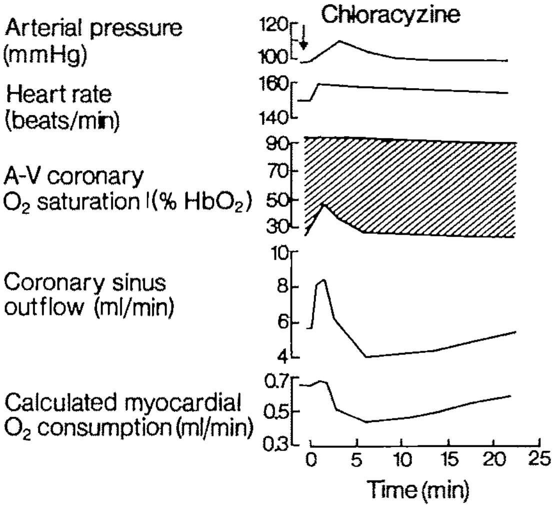


**Figure 1** Effect of chloracyzine (5 mg/kg, i.v.) on myocardial oxygen consumption in open-chest cat.

## Results

### *Open-chest cat*

Chloracyzine induced a decrease in myocardial oxygen consumption (Figure 1). The decrease in oxygen uptake was 15% for a dose of 2 mg/kg and 26% for 5 mg/kg (Table 1). Coronary blood flow increased for 2–4 min and then was reduced in accordance with the level of myocardial oxygen consumption. Coronary sinus oxygen saturation was greatly increased during coronary dilatation. When coronary blood flow was reduced there were no significant changes in oxygen saturation. Despite the

**Table 1** Effects of chloracyzine in the open-chest cat at the time of maximum decrease in myocardial $O_2$ consumption

| | *Chloracyzine 2 mg/kg, i.v. (9)* | | *Chloracyzine 5 mg/kg, i.v. (10)* | |
|---|---|---|---|---|
| | *Control* | *5 min after injection* | *Control* | *7 min after injection* |
| Myocardial $O_2$ consumption (ml/min) | 0.90 ± 0.04 | 0.76 ± 0.05† | 0.86 ± 0.05 | 0.64 ± 0.03‡ |
| Arterial $O_2$ saturation (%HbO$_2$) | 94 ± 4 | 95 ± 3 NS | 96 ± 3 | 96 ± 3 NS |
| Coronary sinus $O_2$ saturation (%HbO$_2$) | 27 ± 2 | 29 ± 2 NS | 28 ± 2 | 31 ± 3 NS |
| Coronary sinus outflow (ml/min) | 8.2 ± 0.4 | 7.0 ± 0.4† | 7.8 ± 0.4 | 6.2 ± 0.5† |
| Mean arterial pressure (mmHg) | 95 ± 6 | 97 ± 7 NS | 90 ± 7 | 95 ± 8 NS |
| Heart rate (beats/min) | 161 ± 8 | 169 ± 9 NS | 165 ± 7 | 180 ± 8* |

Values are mean ± s.e. mean. Number of experiments in parentheses.
* $P<0.05$; † $P<0.01$; ‡ $P<0.001$.

decrease in oxygen uptake after chloracyzine, the heart rate increased and mean arterial pressure was not reduced, in fact, it increased in many instances.

Aminophylline caused a pronounced and almost equal increase in myocardial oxygen consumption and coronary blood flow. Marked coronary dilatation induced by prenylamine was accompanied by an insignificant increase in oxygen uptake.

### *Isolated heart*

Chloracyzine again reduced myocardial oxygen consumption. The decrease was 16% and 30% for doses of 0.2 mg and 0.5 mg respectively (Table 2). Coronary flow was also reduced (after an initial increase for the first 1–2 min) but to a smaller degree than myocardial oxygen consumption which resulted in a significant increase in coronary outflow oxygen content.

## Discussion

In the open-chest preparation, the decrease in myocardial oxygen consumption after chloracyzine was chiefly due to reduction in coronary blood flow, while the reduced A–V $O_2$ difference played a role only in the initial stage. By contrast, the latter was the main factor for reduction in oxygen uptake in the isolated heart. A possible explanation for this difference is impairment, in the latter case, of the mechanism coordinating the coronary blood flow and myocardial oxygen consumption.

Chloracyzine was able to produce a small increase in mean arterial pressure; heart rate increased slightly in the open-chest experiments but not in the isolated heart. These effects can be attributed to the atropine-like action of chloracyzine and to its ability to augment the pressor action of catecholamines. Markova (1963) reported a tendency to increase cardiac output. All these findings indicate that despite the decrease in oxygen consumption, heart activity is at least not reduced. Chloracyzine thus increases the heart efficiency.

It was found previously that chloracyzine increases the coefficient of oxidative phosphorylation in homogenates of the myocardium of rats treated with this drug (Kisin, 1973). Ability of other phenothiazine derivatives to increase the efficiency of oxidative phosphorylation was also reported. Zubovskaya (1960) showed that mepazine and promazine when added to the homogenates of myocardium of the rabbit caused a rise in the P : O coefficient. Improvement in the process of formation of energy-rich phosphates is a possible explanation for the increase in heart efficiency after chloracyzine.

The decrease in myocardial oxygen consumption induced by chloracyzine as well as its spasmolytic properties may be responsible for the positive effect of this drug in angina pectoris (Kisin, 1973). It seems likely that the drug decreases myocardial oxygen uptake through improved efficiency in the use of oxygen. This approach may be of interest as in such a case a reduced oxygen uptake is not associated with reduced heart activity which is often undesirable.

I wish to thank Dr V. V. Buyanov for help in carrying out the experiments.

**Table 2** Effects of chloracyzine in the isolated heart at the time of maximum decrease in myocardial $O_2$ consumption

| | *Chloracyzine 0.2 mg (7)* | | *Chloracyzine 0.5 mg (8)* | |
|---|---|---|---|---|
| | *Control* | *3 min after administration* | *Control* | *5 min after administration* |
| Myocardial $O_2$ consumption (μg/min) | 202 ± 15 | 164 ± 11† | 211 ± 14 | 148 ± 15‡ |
| Coronary inflow $O_2$-content (μg/ml) | 20 ± 2 | 20 ± 2 | 20 ± 2 | 20 ± 2 |
| Coronary outflow $O_2$-content (μg/ml) | 3.2 ± 0.2 | 4.9<0.4‡ | 3.5 ± 0.3 | 7.2 ± 0.7‡ |
| Coronary outflow (ml/min) | 12.1 ± 0.8 | 11.2 ± 0.7 NS | 13.0 ± 0.6 | 11.5 ± 0.8* |
| Heart rate (beats/min) | 120 ± 10 | 116 ± 11 NS | 128 ± 9 | 122 ± 12 NS |

Values are mean ± s.e. mean. Number of experiments in parentheses.
* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

## References

KISIN, I.E. (1973). Drugs for the treatment of angina pectoris. In *Advances in Drug Research*, ed. Kharkevich, D., pp. 271–281. Moscow: Medicina.

KISIN, I.E. & BUYANOV, V.V. (1963). Effect of adrenaline, ephedrine and amphetamine on the tone of the coronary vessels and myocardial oxygen consumption in isolated heart. *Byull. Eksp. Biol. Med.*, **55**, (N2), 68–72. (Cover-to-cover English translation. Plenum Publishing Co., New York.)

MARKOVA, G.A. (1963). Effect of vasodilating drugs on heart work in myocardial infarction. *Farmakol. Toksikol.*, **26**, 164–169.

VIKHLJAEV, J.I. & KAVERINA, N.V. (1959). On the pharmacology of chloracyzine. *Farmakol. Toksikol.*, **19**, (N1), 28–34.

ZHURAVLEV, S.V., GRITSENKO, A.N. & ERMAKOVA, Z.I. (1968). Method of producing 10-(β-diethylaminopropionyl)-2-chlorophenothiazine. British Patent 1,110, 458.

ZUBOVSKAYA, A.M. (1960). The effect of certain phenothiazine derivatives on oxidative phosphorylation in the cardiac muscle of the rabbit. *Byull. Eksp. Biol. Med.*, **49**, (N4), 60–63. (Cover-to-cover English translation. Plenum Publishing Co., New York.)

*(Received January 5, 1976.*
*Revised March 26, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 193–199

# STIMULATION OF PROSTAGLANDIN BIOSYNTHESIS BY DRUGS: EFFECTS *in vitro* OF SOME DRUGS AFFECTING GUT FUNCTION

H.O.J. COLLIER, WENDY J. McDONALD-GIBSON & S.A. SAEED
Research Department, Miles Laboratories Limited, Stoke Poges, Slough, SL2 4LY

1 Low concentrations of several emetic, purgative or irritant drugs in the absence of added co-factors stimulated conversion of arachidonic acid to prostaglandin $E_2$ and $F_{2\alpha}$ by prostaglandin synthetase extracted from bull seminal vesicles (BSV prostaglandin synthetase). Their effect was dependent on concentration and time.

2 Stimulation of BSV prostaglandin synthetase by apomorphine, aloes, tyramine or zingerone was increased several-fold by addition of reduced glutathione to the incubation medium, whereas hydroquinone, a phenolic co-factor of prostaglandin synthetase caused slight depression.

3 From this finding and from the observation that many of the stimulant drugs possess a phenolic group, whereas their inactive relatives lack such a group, it is suggested that these stimulant drugs act as co-factors for prostaglandin synthetase in place of hydroquinone.

4 Aloes, tyramine, ethanol and quipazine also produced a dose-related increase in resting tone of the isolated fundus of the rat stomach. This increase occurred at concentrations comparable to those effective in stimulating BSV prostaglandin synthetase, and was abolished by acetylsalicylate.

5 These findings support the view that certain drugs exert some of their pharmacological effects by stimulating prostaglandin synthetase.

## Introduction

Many parts of the digestive tract release prostaglandins in response to various stimuli (Coceani, Pace-Asciak, Volta & Wolfe, 1967; Bennett, Friedmann & Vane, 1967; Collier, 1974; Herman & Vane, 1975). Administration of exogenous prostaglandins causes emesis, bile reflux, accumulation of water and electrolytes in the lumen of the small intestine, abdominal pain and diarrhoea (Horton, Main, Thompson & Wright, 1968; Misiewicz, Waller, Kiley & Horton, 1969; Matuchansky & Bernier, 1973; Milton-Thompson, Cummings, Newman, Billings & Misiewicz, 1975; Main & Whittle, 1975). These facts led us to investigate the possibility that some drugs exert their effects on the digestive tract through stimulation of prostaglandin biosynthesis in the gut wall or elsewhere in the body. Preliminary experiments, some of which have been briefly reported (Butt, Collier, Gardiner & Saeed, 1974; Collier, McDonald-Gibson & Saeed, 1974; 1975), showed that some emetics, purgatives or flavouring agents stimulate prostaglandin biosynthesis in homogenates of bull seminal vesicles incubated with arachidonic acid We now describe further studies on the more potent stimulants, and investigations of other drugs.

## Methods

### *Enzyme preparation*

Bull seminal vesicles from the slaughterhouse were used fresh or after not more than four weeks storage at −20°C. Vesicles were trimmed of fat and connective tissue, cut into small pieces and homogenized for 1–2 min at full speed in a Waring Blendor in ice-cold 50 mM phosphate buffer at pH 7.4 (referred to subsequently as buffer) containing 1 mM disodium edetate (EDTA). The suspension was strained through cheesecloth and centrifuged for 20 min at 600 *g*. The supernatant was used as the synthetase preparation.

### *Standard test*

After the optimal conditions for prostaglandin biosynthesis and the time during which production increase was linear had been determined, using a procedure already outlined by Collier *et al.*, 1974, the following standard test was adopted. To 0.5 ml of the synthetase preparation arachidonate was added to give a final concentration as sodium salt of 61 µM (20 µg/ml). Appropriate concentrations of test drugs were included in the reaction mixture which was made up to 2 ml with buffer and incubated with shaking at 37°C for 15 minutes. Two ml 0.2 M citric acid and 16 ml ethylacetate were added, and after thorough mixing and centrifugation for 5 min at 600 *g*, 10 ml of the ethylacetate layer was removed, evaporated to dryness *in vacuo* and the residue dissolved in Krebs solution for bioassay of total prostaglandin-like activity. All experiments included controls in which boiled BSV homogenate was used.

For separate assay of E and F prostaglandin-like activities, the dried residue was redissolved in 50 µl of ethanol and spotted quantitatively onto thin-layer silica gel plates, with markers of 2 µg prostaglandin $E_2$ and $F_{2\alpha}$. The dried plate was developed to a distance of about 16 cm in a modified AI (Green & Samuelsson, 1964) solvent system (benzene: dioxane: acetic acid: 50:50:1). After chromatography, the marker spots were visualized by spraying with 10% phosphomolybdic acid in ethanol and exposing to hot air. The areas corresponding to prostaglandins $E_2$ and $F_{2\alpha}$ were scraped into test tubes and eluted with acetone or ethanol. The eluates were dried in a stream of $N_2$ and taken up in Krebs solution for bioassay.

In four experiments, 0.125 µCi [$^3$H]-prostaglandin $E_2$ and 0.125 µCi [$^3$H]-prostaglandin $F_{2\alpha}$ were incubated with unboiled or boiled enzyme for 15 min, extracted, separated by thin-layer chromatography and assayed. In this procedure, <8% of the prostaglandins $E_2$ and $F_{2\alpha}$ was lost during incubation with unboiled enzyme preparation. It was concluded that the increased net prostaglandin production in the presence of test drugs could be attributed largely to increased prostaglandin biosynthesis and not to protection from breakdown.

### *Bioassay*

Bioassay of prostaglandins was performed, with two to four samples of each test solution, on rat stomach fundus strip, superfused at 5 ml/min with Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.6, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.6, gassed with 95% $O_2$ and 5% $CO_2$ and containing a mixture of selective antagonists: hyoscine 0.33 µM, mepyramine 0.35 µM, methysergide 0.57 µM, phenoxybenzamine 0.33 µM and propranolol 11.57 µM. As the product of the enzyme in the conditions of these experiments was largely prostaglandin $E_2$-like, this prostaglandin was used for reference. Some results were expressed as the production ratio, which is the ratio of total prostaglandin production in the presence of drug to that in controls. When prostaglandins $E_2$ and $F_{2\alpha}$ were assayed separately, the rat stomach strip and rat colon were used respectively.

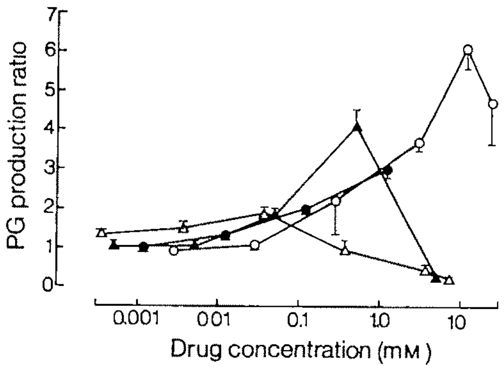


**Figure 1** Concentration-response lines for total prostaglandin (PG) production (assayed as prostaglandin $E_2$) by stimulant drugs in standard test conditions (see Methods section). The prostaglandin production ratio is the ratio of prostaglandin production in the presence of drug to that in its absence. Aloes (●); tyramine (○); capsaicin (△); zingerone (▲). Vertical lines represent standard errors of the mean.

### *Fundus of rat stomach*

To measure the effect of drugs on isolated tissue, a method was adapted from one used previously to study inhibition of prostaglandin synthetase (Bennett, Fox & Stamford, 1973; Collier, 1974). A strip of rat stomach fundus was suspended in oxygenated Krebs solution in an organ bath (10 ml). When the preparation had reached a constant tone, a low concentration of drug was added. The concentration of drug was then doubled and this process continued until tone reached a maximum. The results are expressed as the total concentration to increase tone to 50% maximum ($MT_{50}$).

### *Drugs*

Table 1 lists the drugs tested and the salts used. Colchicine (Sigma), caffeine citrate (BDH), 3-isobutyl-1-methylxanthine (IBMX; Aldrich), theobromine (Sigma), theophylline (Sigma), capsaicin (Sigma) and *Escherichia coli*, strain 0111 B4, lipopolysaccharide W toxin (Difco) were used. Bile was obtained from the gall bladder of freshly killed guinea-pigs. Quipazine is 1-(2-quinolyl)piperazine (Rodriguez & Pardo, 1971). Solutions were made up in water or, if necessary, in dilute HCl or NaOH and serially diluted in buffer for addition to the incubation mixture. [$^3$H]-prostaglandin

$E_2$ (160 Ci/mmol) and [$^3$H]-prostaglandin $F_{2\alpha}$ (20 Ci/mmol) (Radiochemical Centre, Amersham), were diluted in a solution of the unlabelled prostaglandin before use. Concentrations of drugs refer to the acid or base.

Sodium arachidonate was prepared by dissolving arachidonic acid (Sigma, grade 1, 99% pure) in ethanol and diluting with 0.2% w/v sodium carbonate. This solution of sodium arachidonate was diluted in an amber glass container with buffer to 200 μg/ml for use.

Drugs were tested at several dilutions to determine the concentration that stimulates total prostaglandin production by 50% ($SC_{50}$), corresponding to a production ratio of 1.5. Dose-response lines were plotted for drugs in the absence of added hydroquinone or glutathione (standard conditions) that gave a production ratio significantly ($P < 0.05$) more than unity. From this line, the $SC_{50}$ value was derived.

## Results

### *Bull seminal vesicle prostaglandin synthetase*

Many of the drugs produced a concentration-related increase in total prostaglandin production in the standard test conditions, although some active drugs in high concentrations strongly inhibited prostaglandin production assayed as prostaglandin $E_2$ (Figure 1). In Table 1, based on dose-response lines of the type shown in Figure 1, drugs are grouped according to probably relevant effect; potency is expressed as the $SC_{50}$ value derived from the rising phase of the dose-response line and effectiveness as the maximal prosta-

**Table 1** Potency and effectiveness of test drugs as stimulants of prostaglandin (PG) biosynthesis assayed as prostaglandin $E_2$

| *Drug effect* | *Drug* | *$SC_{50}$ ± s.e.* | *$S_{max}$ ± s.e.* | *Most effective concentration tested* |
|---|---|---|---|---|
| Emesis | Apomorphine hydrochloride | 0.027 ± 0.01 | 4.13 ± 0.17 | 0.37 |
| | Codeine phosphate | NS (0.84) | — | — |
| | Heroin hydrochloride | 0.62 ± 0.06 | 2.06 ± 0.25 | 2.36 |
| | Morphine sulphate | 0.17 ± 0.06 | 5.43 ± 1.45 | 3.50 |
| | Naloxone hydrochloride | 0.19 ± 0.04 | 5.23 ± 0.83 | 2.75 |
| Diarrhoea | Aloes B.P. | 9.623 ± 1.67* | 2.99 ± 0.13 | 500* |
| | Bisacodyl | NS (0.14) | — | — |
| | Colchicine | 6.76 ± 0.25 | 2.80 ± 1.28 | 7.5 |
| | Danthron | NS (0.12) | — | — |
| | Oxyphenisatin acetate | NS (0.16) | — | — |
| | Phenolphthalein diphosphate | 1.85 ± 0.72 | 1.50 ± 0.14 | 1.85 |
| Migraine | α-Phenylethylamine hydrochloride | NS (24.8) | — | — |
| | β-Phenylethylamine hydrochloride | NS (7.96) | — | — |
| | Tyramine hydrochloride | 0.35 ± 0.10 | 6.08 ± 0.55 | 11.52 |
| Inhibition of phospho-diesterase | Caffeine citrate | 1.35 ± 0.86 | 2.25 ± 0.19 | 10.30 |
| | IBMX | 1.44 ± 0.76 | 1.76 ± 0.16 | 2.25 |
| | Theobromine | Circa 3.6 | 1.49 | 3.6 |
| | Theophylline | 3.12 ± 2.20 | 2.09 ± 0.61 | 11.09 |
| Irritation | Capsaicin | 0.006 ± 0.002 | 1.83 ± 0.22 | 0.036 |
| | Zingerone | 0.041 ± 0.01 | 4.05 ± 0.45 | 0.52 |
| | Ethanol | 2.5 ± 0.164† | 1.50 ± 0.17 | 2.5† |
| | Acetaldehyde | NS (910) | — | — |
| | *E. coli* toxin | NS (250)* | — | — |
| | Guinea-pig bile | 5.45 ± 2.0† | 1.66 ± 0.11 | 15.0† |
| | Quipazine maleate | 0.07 ± 0.04 | 1.6 ± 0.08 | 0.47 |
| Stimulation of PG synthetase | Glutathione | 0.021 ± 0.009 | 9.09 ± 0.77 | 1.3 |
| | Hydroquinone | 0.093 ± 0.044 | 1.95 ± 0.12 | 0.33 |
| | Isoprenaline sulphate | 0.12 ± 0.024 | 4.47 ± 0.56 | 1.35 |

The $SC_{50}$ is the lowest mM concentration of drug giving a total prostaglandin production that is 50% higher than controls. $S_{max}$ is the prostaglandin production at the most effective stimulatory concentration of drug used, expressed as the ratio of total prostaglandin produced in the presence of drug to that in its absence. NS (0.84) etc., not stimulant at maximal concentration used, 0.84 mM. * μg/ml; † %v/v.

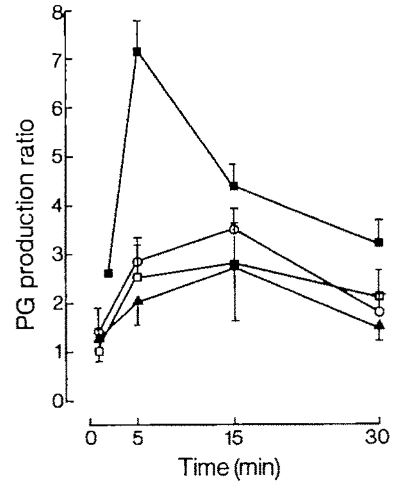


**Figure 2** Time-response lines for stimulant drugs in the standard conditions of test. Response is the ratio of total prostaglandin (PG) production reached at a given point in time in incubates containing drug to that in incubates without drug. Apomorphine 0.37 mM (■); colchicine 7.8 mM (□); tyramine 3.6 mM (O); zingerone 0.52 mM (▲). Vertical lines represent standard errors of the means.

glandin production ratio obtained. The slopes of the rising phases of the dose-response lines of all drugs in Table 1 had a value of $P<0.05$, and eleven of these slopes had values of $P<0.01$. Apomorphine and morphine, which have emetic effects, had high activity, and except for codeine their relatives were also active. Activity was shown by aloes, colchicine and phenolphthalein, which have purgative effects, but not by bisacodyl, danthron or oxyphenisatin. Some food substances suspected of causing migraine (Hanington, 1967; Sandler, Youdim & Hanington, 1974), were active (tyramine, present in cheese and theobromine, present in chocolate). The 'hot' flavourings capsaicin, from chilli, and zingerone, from ginger, were also active. Ethanol was active but its metabolite, acetaldehyde, was inactive. The phenylethylamines, in chocolate, were inactive. Quipazine showed high potency but moderate effectiveness. That glutathione or hydroquinone stimulated prostaglandin synthetase indicates the standard test medium contained less than optimal amounts of co-factors.

Figure 2 gives the time-response lines of some active drugs. The onset of stimulation was rapid and the increase of prostaglandin production was roughly linear up to approximately 5 to 15 min, and then fell.

In other experiments, we separated by thin-layer chromatography and estimated separately the prostaglandin $E_2$- and $F_{2\alpha}$-like material produced in control incubates and in the presence of apomorphine, morphine, naloxone, tyramine or capsaicin. In all incubates, both prostaglandins were regularly detected, prostaglandin $E_2$ always exceeding $F_{2\alpha}$ by several-fold in amount.

Acetylsalicylate, an inhibitor of prostaglandin synthetase, inhibited the stimulation of total prostaglandin biosynthesis by apomorphine, tyramine, capsaicin, glutathione or hydroquinone (Table 2). The $IC_{50}$ value of acetylsalicylate ranged from 2.0 mM for hydroquinone to 6.4 mM for tyramine.

To explore the mechanism of stimulant action, we tested some of the active drugs in the presence of glutathione or hydroquinone, the co-factors commonly used to stimulate prostaglandin synthetase (Nugteren, Beerthuis & Van Dorp, 1966; Samuelsson, 1967). Hydroquinone somewhat lessened the stimulation of prostaglandin production by two concentrations of aloes, apomorphine, tyramine or zingerone, whereas glutathione markedly enhanced their stimulant action (Table 3). Time-response lines also showed that glutathione strongly enhanced the effect of apomorphine, but hydroquinone did not.

### *Fundus of rat stomach*

Aloes, tyramine, zingerone, ethanol, quipazine, glutathione and hydroquinone were tested for ability to increase the tone of the isolated fundus of rat

**Table 2** Concentrations of acetylsalicylate required to inhibit by 50% ($IC_{50}$) the stimulation of total prostaglandin production by various drugs

| *Stimulant (mM)* | *No. of expts* | *Production ratio in absence of acetylsalicylate* | *$IC_{50}$ of acetylsalicylate (mM)* |
|---|---|---|---|
| Apomorphine (0.037) | 4 | 2.39 | 2.95 |
| Apomorphine (0.37) | 3 | 4.09 | 2.95 |
| Capsaicin (0.0037) | 2 | 1.28 | 3.69 |
| Capsaicin (0.037) | 2 | 1.70 | 2.26 |
| Glutathione (0.325) | 3 | 5.09 | 2.4 |
| Hydroquinone (0.325) | 3 | 1.95 | 2.0 |
| Tyramine (3.64) | 3 | 4.0 | 6.4 |

stomach. Of these, aloes, tyramine, ethanol and quipazine produced a dose-related increase in tone (Table 4). Acetylsalicylate (5.0 mM) abolished the increase produced by each drug at above its $MT_{50}$ concentration, except tyramine, of which a dose lower than the $MT_{50}$ was fully inhibited.

## Discussion

Few of the drugs in Table 1 that stimulated prostaglandin biosynthesis appear to have been shown by others to be potent stimulants of this process *in vitro*. After incubation for several days in the presence of 0.1 µg/ml colchicine, cultures of synovial tissue from patients with rheumatoid arthritis produced more than ten times as much prostaglandin as controls (Robinson, Smith, McGuire & Levine, 1975). However, when tested over a 15 min incubation period, colchicine had a relatively low potency and effectiveness (Table 1). The toxic effects of colchicine in man include nausea, emesis, abdominal pain, diarrhoea and a burning sensation of skin and mucous membranes; these appear after a pronounced latency (Woodbury & Fingle, 1975) and this is consistent with the possibility that its action is indirect.

Despite the ability of *Escherichia coli* endotoxin to produce vomiting and diarrhoea *in vivo* (Thomas, 1954; Collier, 1974), it failed to stimulate prostaglandin biosynthesis when incubated for 15 min with BSV homogenate (Table 1). This failure is consistent with the findings that only after a latency of 2 h did *E. coli* endotoxin *in vivo* cause emesis and diarrhoea (Collier, 1974) and that prostaglandin synthesis is stimulated only by toxin administered *in vivo* (Herman & Vane, 1975).

How do drugs stimulate prostaglandin biosynthesis by BSV homogenate? Since the homogenate in the standard test conditions did not appreciably degrade added radio-labelled prostaglandins $E_2$ or $F_{2\alpha}$, blockade of degradation of synthesized prostaglandins could not explain their increased amounts. It seems

**Table 3** Effects of glutathione (GSH) or hydroquinone (HQ), both 0.13 mM, on stimulation of prostaglandin biosynthesis by aloes, apomorphine, tyramine or zingerone in bull seminal vesicle homogenate

| | | *Addition* | |
|---|---|---|---|
| *Drug* | *None* | *GSH* | *HQ* |
| None | 1.01 | 3.39 | 2.12 |
| Apomorphine 0.037 mM | 1.61 | 6.10 | 1.97 |
| Apomorphine 0.37 mM | 4.58 | 9.93 | 3.83* |
| None | 1.06 | 4.24 | 3.14 |
| Aloes 50 µg/ml | 1.82 | 6.72 | 3.05 |
| Aloes 500 µg/ml | 2.53 | 9.41 | 1.74* |
| None | 0.87 | 4.10 | 2.15 |
| Tyramine 0.36 mM | 1.28 | 6.24 | 2.17 |
| Tyramine 3.6 mM | 3.05 | 11.94 | 2.59* |
| None | 1.12 | 6.2 | 3.32 |
| Zingerone 0.051 mM | 2.15 | 9.16 | 4.21 |
| Zingerone 0.51 mM | 3.86 | 9.96 | 5.50 |

All results are expressed as µg prostaglandin $E_2$ equivalents produced. Values are the means of two independent experiments. * Below control levels.

**Table 4** Potency of some stimulant drugs in increasing the tone of the rat stomach fundus; and the highest concentration of drug at which 5 mM acetylsalicylate (ASA) abolished the tone increase

| *Drug* | *$MT_{50}$ ± s.e.* | *Max. concn inhibited by ASA* |
|---|---|---|
| Aloes B.P. | 9.0 ± 1.9 µg/ml | 31.8 ± 6.1 µg/ml |
| Tyramine | 0.35 ± 0.02 mM | 0.23 ± 0.003 mM |
| Ethanol | 4.2 ± 1.0% v/v | 7.0 ± 0.7% v/v |
| Quipazine | 0.017 ± 0.0025 mM | 0.03 ± 0 mM |

$MT_{50}$ is the total concentration of the test drug to increase tone by 50% of the maximal increase. Each value is the mean of four experiments.

unlikely that the stimulant drugs acted by releasing endogenous arachidonic acid through the activation of phospholipase A, because the medium contains both added arachidonic acid, and EDTA, which inhibits phospholipase A (Roy, 1975).

The stimulant drug might act as a co-factor, supplementing a sub-optimal co-factor concentration in the medium. Lessening by hydroquinone and enhancement by glutathione of the ability of several stimulant drugs to increase prostaglandin biosynthesis (Table 3) fits this view. These considerations lead us to think that the effects observed were mainly due to stimulation of prostaglandin synthetase.

Most drugs showing stimulant activity (Table 1) were phenolic, whereas the only ineffective phenolic drug was danthron. Furthermore, where a pair of drugs differed only in the presence or absence of a phenolic group, such as morphine and codeine, or tyramine and phenylethylamine, only the phenol was active. Comparable results were obtained by Pace-Asciak (1972) with tyramine and phenylethylamine, using rat stomach prostaglandin synthetase. The unexpected activity of heroin may perhaps be explained by some hydrolysis of the acetyl group in the test conditions (heroin is unstable in aqueous solution), and the inactivity of danthron by its low water solubility. We suggest that the stimulant effects on prostaglandin synthesis of certain drugs, described above, depends on the presence of a phenolic group although this group could not be the only determinant of stimulant activity. Potency and effectiveness among phenols would be expected to vary in relation to other aromatic ring substituents of the molecule. Such drugs may thus be thought to play the part of 'phenolic activator' (Smith & Lands, 1971) of prostaglandin synthetase, which may also be played *in vivo* by catecholamines or 5-hydroxyindoles (Sih, Takeguchi & Foss, 1970; Takeguchi, Kohno & Sih, 1971).

It seemed desirable that observations made on a broken cell preparation of bull seminal vesicles should be extended to tissue that was derived from the gastrointestinal tract and contained intact functioning cells. We therefore tested the effects of some active drugs on the resting tone of an isolated strip of the fundus of rat stomach, which is regarded as due to intramural generation of prostaglandins (Eckenfels & Vane, 1972). The test was partly successful, in that four of seven stimulant drugs increased the resting tone of the fundus (Table 4) and, where the fundus responded, the effective concentration was comparable with that in BSV homogenate. The inhibition by acetylsalicylate of a considerable part of the increase of tone suggests that prostaglandin biosynthesis was involved.

That ethanol was effective in both tests is consistent with the recent conclusion of Karppanen & Puurunen (1976) that ethanol inhibits acid gastric secretion in the rat, at least partly through the increased synthesis of prostaglandins. Quipazine, which was active in both tests, has the side-effects of nausea, salivation, emesis, retching, gastric discomfort, abdominal distension, flatulence, colic, diarrhoea, tenesmus and headache (J.E. Villarreal, personal communication).

Our findings point to a hitherto little appreciated facet of drug action: certain drugs stimulate prostaglandin synthetase and this may mediate some of the drugs' effects. Since prostaglandin synthetase occurs in the gut (Pace-Asciak & Wolfe, 1970) and prostaglandin precursors are present in foods, the local concentration of drug might well be enough to stimulate prostaglandin biosynthesis, particularly in the presence of prostaglandin precursors in dietary fats, and thus contribute to symptoms such as dyspepsia, diarrhoea, headache and a sensation of heat in mucous membranes.

We thank Miss J. Copas, Mrs J. Cuthbert, Mr B. Hawkins, Mr I. Richards and Mr C. Shah for technical help.

## References

BENNETT, A., FOX, C.F. & STAMFORD, I.F. (1973). Inhibition of prostaglandin synthesis by Benorylate. *Rheumatology and Rehabilitation* (Suppl.), 101–105.

BENNETT, A., FRIEDMANN, C.A. & VANE, J.R. (1967). Release of prostaglandin $E_1$ from the rat stomach. *Nature, Lond.*, **216**, 873–876.

BUTT, A.A., COLLIER, H.O.J., GARDINER, P.J. & SAEED, S.A. (1974). Effects on prostaglandin biosynthesis of drugs affecting gastrointestinal function. *Gut*, **15**, 344.

COCEANI, F., PACE-ASCIAK, C., VOLTA, F. & WOLFE, L.S. (1967). Effect of nerve stimulation on prostaglandin formation and release from the rat stomach. *Amer. J. Physiol.*, **213**, 1056–1064.

COLLIER, H.O.J. (1974). Prostaglandin synthetase inhibitors and the gut. In *Prostaglandin Synthetase Inhibitors*. ed. Robinson, H.J. & Vane, J.R. pp. 121–133. New York: Raven.

COLLIER, H.O.J., McDONALD-GIBSON, W.J. & SAEED, S.A. (1974). Apomorphine and morphine stimulate prostaglandin biosynthesis. *Nature, Lond.*, **252**, 56–58.

COLLIER, H.O.J., McDONALD-GIBSON, W.J. & SAEED, S.A. (1975). Stimulation of prostaglandin biosynthesis by capsaicin, ethanol and tyramine. *Lancet*, **i**, 702.

ECKENFELS, A. & VANE, J.R. (1972). Prostaglandins, oxygen tension and smooth muscle tone. *Br. J. Pharmac.*, **45**, 451–462.

GRÉEN, J. & SAMUELSSON, B. (1964). Prostaglandins and related factors: XIX. Thin-layer chromatography of prostaglandins. *J. Lipid Res.*, **5**, 117–120.

HANINGTON, E. (1967). Preliminary report on tyramine headache. *Br. med. J.*, **2**, 550–551.

HERMAN, A.G. & VANE, J.R. (1975). Endotoxin and production of prostaglandins by the isolated rabbit jejunum. Influence of indomethacin. *Arch. Int.*

*Pharmacodyn. Thér.*, **213**, 328–329.

HORTON, E.W., MAIN, I.H.M., THOMPSON, C.J. & WRIGHT, P.M. (1968). Effect of orally administered prostaglandin $E_1$ on gastric secretion and gastrointestinal motility in man. *Gut*, **9**, 655–658.

KARPPANEN, H. & PUURUNEN, J. (1976). Ethanol, indomethacin and gastric secretion in the rat. *Europ. J. Pharmac.*, **35**, 221–223.

MAIN, I.H.M. & WHITTLE, B.J.R. (1975). Potency and selectivity of methyl analogues of prostaglandin $E_2$ on rat gastrointestinal function. *Br. J. Pharmac.*, **54**, 309–317.

MATUCHANSKY, C. & BERNIER, J.-J. (1973). Effect of prostaglandin $E_1$ on glucose, water and electrolyte absorption in the human jejunum. *Gastroenterology*, **64**, 1111–1118.

MILTON-THOMPSON, G.J., CUMMINGS, J.H., NEWMAN, A., BILLINGS, J.A. & MISIEWICZ, J.J. (1975). Colonic and small intestinal response to intravenous prostaglandin $F_{2\alpha}$ and $E_2$ in man. *Gut*, **16**, 42–46.

MISIEWICZ, J.J., WALLER, S.L., KILEY, N. & HORTON, E.W. (1969). Effect of oral prostaglandin $E_1$ on intestinal transit in man. *Lancet*, **i**, 648–651.

NUGTEREN, D.H., BEERTHUIS, R.K. & VAN DORP, D.A. (1966). The enzymatic conversion of *all-cis* 8, 11, 14-eicosatrienoic acid intro Prostaglandin $E_1$. *Recl. Trav. chim. Pays-Bas Belg.*, **85**, 405–419.

PACE-ASCIAK, C. (1972). Prostaglandin synthetase activity in the rat stomach fundus: activation by L-norepinephrine and related compounds. *Biochim. biophys. Acta*, **280**, 161–171.

PACE-ASCIAK, C. & WOLFE, L.S. (1970). Biosynthesis of prostaglandin $E_2$ and $F_{2\alpha}$ from tritium-labelled arachidonic acid by rat stomach homogenates. *Biochim. biophys. Acta*, **218**, 539–542.

ROBINSON, D.W., SMITH, H., McGUIRE, M.B. & LEVINE, L. (1975). Prostaglandin synthesis by rheumatoid synovium and its stimulation by colchicine. *Prostaglandins*, 67–85.

RODRIGUEZ, R. & PARDO, E.G. (1971). Quipazine, a ı type of anti-depressant agent. *Psychopharmacolc (Berl.)*, **21**, 89–100.

ROY, A.C. (1975). Biochemical studies on the mode of ac of disodium cromoglycate and related substances. P Thesis, Brunel University.

SAMUELSSON, B. (1967). Biosynthesis and metabolisn prostaglandins. *Prog. Biochem. Pharmac.*, **3**, 59–70.

SANDLER, M., YOUDIM, M.B.H. & HANINGTON, E. (19 A phenylethylamine oxidising defect in migraine. *Nat Lond.*, **250**, 335–337.

SIH, C.J., TAKEGUCHI, C. & FOSS, P. (1970). Mechanisn prostaglandin biosynthesis. III. Catecholamines serotonin as co-enzymes. *J. Amer. Chem. Soc.*, **92**, 66

SMITH, W.L. & LANDS, W.E.M. (1971). Stimulation blockade of prostaglandin biosynthesis. *J. biol. Ch* **246**, 6700–6702.

TAKEGUCHI, C., KOHNO, E. & SIH, C.J. (19 Mechanism of prostaglandin biosynthesis. Characterization and assay of bovine prostaglar synthetase. *Biochemistry*, **10**, 2372–2376.

THOMAS, L. (1954). The physiological disturbar produced by endotoxins. *Ann. Rev. Physiol.*, 467–490.

WOODBURY, D.M. & FINGLE, E. (1975). Analgesics antipyretics. In *The Pharmacological Basis Therapeutics*, 5th edition. ed. Goodman, L.S. & Giln A. pp. 325–358. London: Collier–Macmillan.

*(Received January 9, 19*
*Revised April 26, 19*

*Br. J. Pharmac.* (1976), **58**, 201–209

# BRADYKININ AND FUNCTIONAL VASODILATATION IN THE SALIVARY GLAND

S.H. FERREIRA[1]
Department of Pharmacology, Royal College of Surgeons, Lincoln's Inn Fields, London WC2A 3PN
L.H. SMAJE
Department of Physiology, University College London, Gower Street, London WC1E 6BT

1 Blood flow through the submandibular gland of the dog was measured and the venous effluent monitored for potential mediators of the functional vasodilatation by passing it over a series of assay tissues.

2 On chorda lingual nerve stimulation there was salivation, an increased blood flow and the release of a bradykinin-like substance (kinin) into the venous effluent.

3 In about half of the preparations, increasing the frequency of stimulation from 2 to 10 Hz led to an increased output of kinin, whereas in the others successive stimulations led to a decreasing output of kinin in the face of normal secretory and vascular responses.

4 Following the administration of atropine, the vasodilatation persisted, salivation was abolished and after several stimulations kinin release could no longer be detected.

5 Release of prostaglandin did not appear to be responsible for the vasodilatation.

6 It is concluded that neither kallikrein nor kinin is the main mediator of parasympathetic vasodilatation in the salivary gland.

## Introduction

Stimulation of the parasympathetic nerve supply to the submandibular gland in dogs and cats leads to salivation and an increase in the blood flow, but only the salivation is blocked by atropine (Heidenhain, 1872). This observation suggested that there were specific parasympathetic vasodilator nerve fibres resistant to atropine, but Ungar & Parrot (1936) suggested that kallikrein, present in the submandibular saliva may mediate the effects of the nervous stimulation. Hilton & Lewis (1955a; 1955b; 1956) provided experimental support for this thesis and extended it to functional vasodilatation in other organs. However, their views were subjected to criticism, (Bhoola, Morley, Schachter & Smaje, 1965; Schachter & Beilenson, 1967; Beilenson, Schachter & Smaje, 1968; Skinner & Webster, 1968; Darke & Smaje, 1973). A particular problem was that neither salivary kallikrein nor kinin could be found in the venous effluent of secreting glands. It was possible, however, that bradykinin released during activation of the gland, was not detected because of the very short half-life of the peptide in blood and tissues (Nobili, 1965; Ferreira & Vane, 1967b), or that any kallikrein or kinin released was eliminated in the lymph (Hilton & Lewis, 1955b).

A rapid method for the estimation of bradykinin in blood is thus required and in a preliminary report Ferreira, Corrado & Rocha e Silva (1969) have shown that the blood-bathed organ technique (Vane, 1964) could be used for the detection of kinin in the venous effluent of the salivary gland. In the present paper we have used the same technique and extended these observations. The destruction of kinins by the blood was prevented by the use of synthetic penta and nona bradykinin-potentiating peptides ($BPP_{5a}$ and $BPP_{9a}$) which are two of the bradykinin-potentiating peptides originally described in the venom of *Bothrops jararaca* (Ferreira, 1965; Greene, Camargo, Kreiger, Stewart & Ferreira, 1972). Potentiation of the contractions of the assay tissues by BPP was taken as confirmation of bradykinin release. We have used additional isolated tissues in an attempt to detect substances other than kinins (e.g. prostaglandins) which could mediate the atropine-resistant vasodilatation caused by chorda tympani nerve stimulation.

---

[1] Present Address: Faculdade de Medicina de Ribeirao Preto, USP, Ribeirao Preto, Brazil.

## Methods

Seventeen dogs of various breeds and either sex, weighing between 17 and 35 kg were used. Anaesthesia was induced by intravenous pentobarbitone sodium (Nembutal, Abbot) 30 mg/kg given via a foreleg vein and supplemented as required via a cannula which was subsequently placed in the femoral vein. To facilitate respiration, a cuffed endotracheal tube was inserted after induction of anaesthesia. Blood pressure was monitored from the femoral artery with a S.E. 4–28 pressure transducer and registered on a Watanabe pen recorder.

### *Blood flow*

The venous drainage of the gland was isolated and other veins draining into the external jugular vein were tied off. Thermocautery was used extensively in order to produce a bloodless field. The external jugular vein was cannulated and drops of blood passed through a closed drop counter. The outflow from the counter went to the stem of a Y tube. One limb of the tube joined a cannula placed in the external jugular vein facing towards the heart, and the other went to a roller pump which supplied the assay tissues. This arrangement ensured a constant supply of 10 ml per min of blood to the assay tissues. When salivary gland blood flow was less than 10 ml/min (e.g. during resting conditions) the balance required was pumped from the external jugular vein; if the glandular blood flow exceeded 10 ml/min (e.g. during maximal vasodilatation) any excess passed into the external jugular vein.

The output from the drop counter was either passed to a rate meter or the individual drops were integrated and the counter discharged at regular intervals with a clock (see Darke & Smaje, 1972).

After the dissection the preparation was left for 30–60 min before beginning blood flow recording, at which time heparin (1000 i.u./kg i.v., Boots) was given.

Intra-arterial injections were made via a cannula passed retrogradely into the lingual artery so that its tip lay at the junction of the lingual artery and the external carotid artery.

### *Nerve stimulation*

The chorda lingual nerve was freed from the connective tissue dorsal to the mylohyoid muscle and separated from the hyoglossus muscle. The nerve was cut as far rostral as possible and stimulated with a fluid electrode at supramaximal voltage (10–20 V) with square wave pulses at a frequency of 2–20 Hz and of 1 ms duration.

The submandibular duct was cannulated distal to the point at which it was crossed by the lingual nerve, using polythene tubing of 2–3 mm external diameter.

### *Assay method*

Variations in the concentration of bradykinin-like substance (kinin) or prostaglandins in the venous effluent were measured continuously by the blood-bathed organ technique (Vane, 1964) with 4 tissues in series: (1) cat jejunum (CJ) or terminal ileum (CTI), or kitten terminal ileum (KTI) (Ferreira & Vane, 1967a; Ferreira, Ng & Vane, 1973), (2) rat stomach strip, (3) rat colon and (4) chick rectum. The cat and kitten tissues contract in the presence of bradykinin; the terminal ileum responds to histamine as well but this was prevented by pretreatment with mepyramine (see below). The rat stomach strip (Vane, 1957), rat colon (Regoli & Vane, 1964) and chick rectum (Mann & West, 1950) respond to prostaglandins. The isolated tissues were pretreated with antagonists of adrenaline, 5-hydroxytryptamine, acetylcholine and histamine (Gillmore, Vane & Wyllie, 1968), so that the only known agonist which contracts the cat tissues was bradykinin (Ferreira & Vane, 1967a), and prostaglandins were the only substances known to contract all three of the other tissues (Gillmore *et al.*, 1968).

An increase in oxygen tension is known to lead to an increased tension in isolated tissues. The $P\text{O}_2$ in the venous effluent was higher during stimulation than at rest and the resulting organ contractions had to be distinguished from those due to release of potential mediators (see Results section). Oxygenation of the venous effluent before it superfused the tissues led to inconsistent results, so it was omitted. Contractions of the assay tissues were monitored with auxotonic levers, attached to 'Harvard' smooth muscle transducers and displayed on a multi-channel recorder (Watanabe). The overall amplification of the system was four to eightfold. The isolated assay tissues were suspended in polypropylene chambers and superfused in series, first with Kreb's solution, while the gland was being prepared and then with the venous effluent from the gland. Blood reached the tissues 30–50 s after leaving the gland.

Test substances (bradykinin, Sandoz; purified hog pancreatic kallikrein, Bayer; prostaglandin $E_1$ and $E_2$, Upjohn; histamine acid phosphate, Burroughs Wellcome; adenosine diphosphate, Sigma; bradykinin potentiating pentapeptide, $BPP_{5a}$ and bradykinin potentiating nona peptide $BPP_{9a}$ kindly supplied by L. Greene, Brookhaven National Laboratories, U.S.A.) were injected into the tubing leaving the gland.

## Results

### *Kinin release*

In 13 experiments the cat tissues used for bradykinin assay responded to concentrations of the peptide of 2–8 ng per ml of bathing blood. They were insensitive

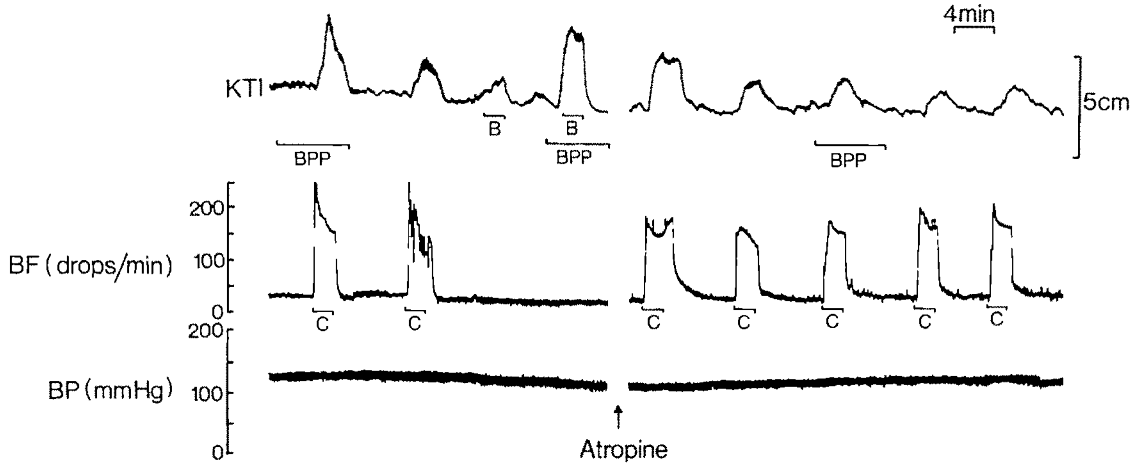


Figure 1 Vascular response and release of kinin following chorda lingual nerve stimulation before and after atropine. The tracings show contractions of kitten terminal ileum (KTI); submandibular gland blood flow (BF); and blood pressure, mmHg (BP). Before atropine, KTI contractions in response to chorda stimulation at a frequency of 5 Hz (C) and to bradykinin 1 ng/ml (B), were enhanced by bradykinin potentiating penta-peptide (BPP$_{5a}$), 2 µg/ml. BPP$_{5a}$ and bradykinin were added to the blood leaving the gland on its way to superfusing the tissues. After atropine (250 µg/kg i.v.) BPP$_{5a}$ did not potentiate the KTI response to chorda stimulation.

to bradykinin in 4 experiments and only the responses to chorda stimulation of the other assay organs (rat colon, chick rectum, rat stomach strip) were studied. In 10 of the 13 dogs, chorda lingual nerve stimulation for 2 min at frequencies of 2–10 Hz led to salivation and vasodilatation, and to contraction of the cat tissues (see left side of Figure 1 and Figure 3). These contractions were not due to a higher oxygen tension in the venous effluent during gland activity because this would have caused contraction of all the assay tissues, which was not observed. The rat colon (not shown in Figures 1 and 3) relaxed following each stimulation period, whereas it contracted when the $P\text{O}_2$ of the superfusing blood was raised by changing from the venous effluent of the gland to femoral arterial blood (see Figure 6).

Addition of bradykinin-potentiating peptide (BPP) to the blood leaving the gland potentiated the responses (Figure 1) of the cat tissues to bradykinin and to the substance released on chorda stimulation in 3 experiments with BPP$_{5a}$ and 4 with BPP$_{9a}$ (1–10 µg/ml). This suggests that chorda stimulation induced release of kallikrein or kinin from the gland.

In 5 of the 10 experiments in which kinin release was detected, the vasodilator response in the gland remained steady with successive chorda lingual nerve stimulation periods, although the output of kinin diminished. In the other 5 both the vasodilatation and the kinin release remained similar to the initial values for several stimulation periods: in these experiments raising the frequency of stimulation from 2 to 10 Hz increased both vasodilatation and kinin release (Figure 2). Thus in the range of stimulus frequencies used, both responses were graded and observed vasodilatation was accompanied by detectable amounts of kinin.

### *Intra-arterial infusion of bradykinin potentiating peptide*

If the bradykinin released during activation of the gland were responsible for the vasodilatation, the latter should be potentiated by BPP. In two experiments, infusion of BPP$_{9a}$ (10 µg/min) into the lingual artery did not affect the vascular responses to chorda lingual nerve stimulation at 2 and 10 Hz but did enhance the contractions of the kitten terminal ileum (KTI). Intra-arterial infusion of bradykinin also produced vasodilatation and subsequently KTI contraction and systemic hypotension. The vasodilatation was enhanced by BPP in one experiment and the systemic hypotension and tissue contractions were increased in both experiments.

### *Effect of atropine*

Atropine (0.2–1 mg/kg, i.v.) blocked the secretory response to chorda stimulation, but not the vasodilator response. The first vasodilator response to chorda stimulation after atropine was usually greater and more prolonged than that found before atropine, and this was accompanied by a greater than normal

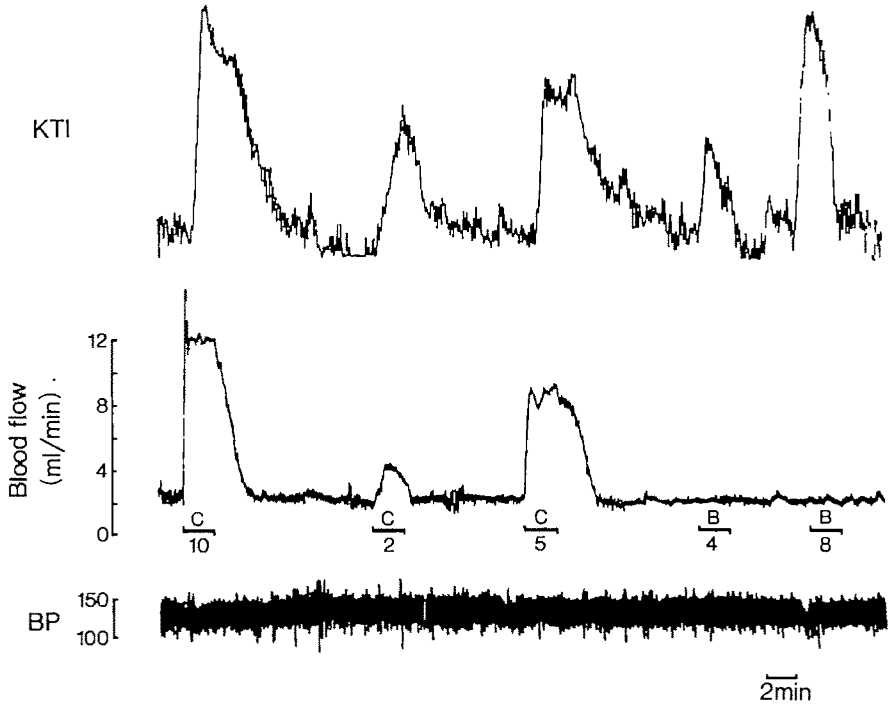


**Figure 2** Effect of changing the frequency of chorda stimulation on submandibular blood flow and on the contractions of the kitten terminal ileum (KTI). C=chorda stimulation at 2, 5 or 10 Hz; B=bradykinin 4 or 8 ng/ml of blood superfusing the KTI.

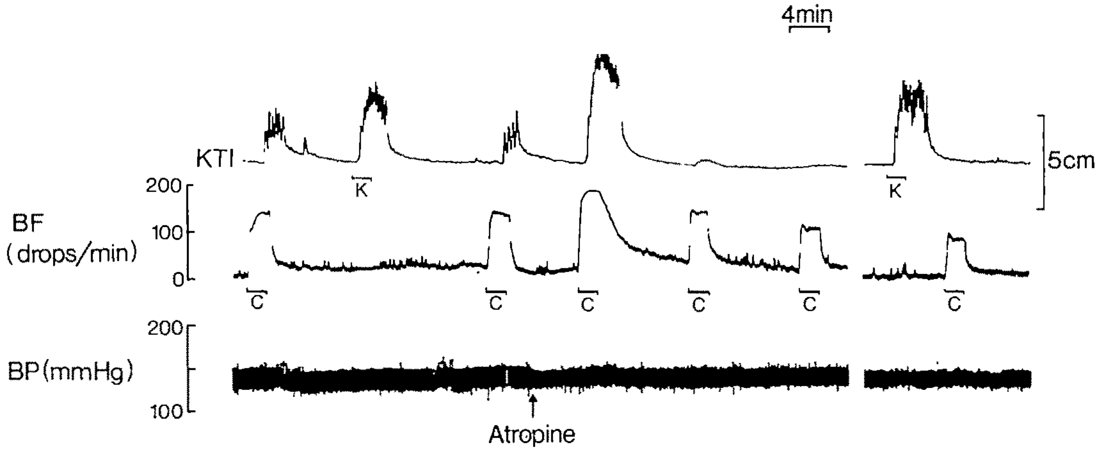


**Figure 3** Vascular response and release of kinin following chorda lingual nerve stimulation. Abbreviations as in Figure 1. The output of kinin and the vascular response to chorda stimulation, 10 Hz (C) were both increased immediately after atropine, (200 μg/kg i.v.). With repeated stimulation, vasodilatation returned to the control level but kinin release diminished to zero. Atropine, if anything, increased the sensitivity of the KTI as the response to kallikrein (K) 60 μu/ml was greater than to K 100 μu/ml given before atropine.

release of kinin-like material (Figures 1 and 3). In some cases the delayed return to the baseline was the most obvious effect.

In 4 experiments the cat tissue contractions diminished with successive stimulation periods and the vascular responses returned towards or were even below the control values (Figure 3). In two of these experiments BPP was administered during the stimulation period but still no kinin-like material was detected. In six other animals chorda stimulation led to contraction of the cat assay tissues even after four or five stimulation periods, but the tissue contractions were not significantly potentiated by BPP (Figures 1 and 4). Figure 4 also shows that atropine did not diminish the contractions of the cat tissue in response to bradykinin, nor affect the increased contraction found with simultaneous infusion of BPP and kinin. The cat tissue contractions remaining after atropine were unlikely to be due to a change in $P\text{O}_2$ as the other assay tissues did not contract at the same time.

A second dose of atropine did not lead to a further increase in kinin release or an increased vasodilator response to stimulation of the chorda lingual nerve.

### *Effect of increasing interstitial kinin concentration*

Kallikrein concentration in the interstitium may be raised by stimulation of the chorda with the excretory duct clamped, or by retrograde infusion of kallikrein (e.g. in saliva) into the salivary duct. These manoeuvres should increase blood flow above normal if kallikrein mediates the vasodilatation.

In three experiments the salivary duct was clamped during stimulation of the chorda lingual nerve before the administration of atropine. Clamping the duct invariably enhanced the effect of chorda stimulation on the kinin released into the blood but not on the vasodilator response.

Retrograde infusion of saliva into the submandibular gland duct was performed in 4 experiments when there was no release of kinin on chorda stimulation after atropine. On stimulation of the chorda lingual nerve substantial amounts of kinin were released in all cases (Figures 5a and b). The vasodilator response was increased in one experiment (Figure 5a), reduced in two (e.g. Figure 5b) and unchanged in one.

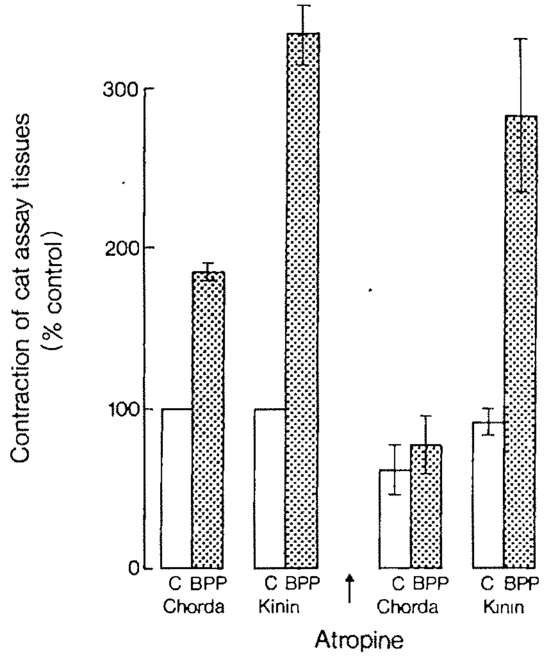


**Figure 4** Effect of bradykinin potentiating peptide (BPP) on contractions of cat or kitten terminal ileum or cat jejunum in response to chorda stimulation or to the addition of bradykinin or kallikrein to the superfusing fluid. The six experiments are shown in which atropine did not abolish contractile responses to chorda stimulation. Control responses (C) were elicited by standard stimuli (5 or 10 Hz, supramaximal voltage) and standard kinin doses, and the effects of BPP expressed as a % of these responses. Mean values are shown; Vertical lines show s.e. mean. Paired *t*-tests indicated that before atropine BPP significantly increased tissue contractions induced by chorda stimulation and by bradykinin ($P<0.01$). After atropine, BPP significantly increased the effect of kinin ($P<0.01$), but not of chorda stimulation. There was no significant difference between the BPP-enhanced responses to kinin before and after atropine, but the effects of chorda stimulation were significantly reduced ($P<0.01$).

### *Release of other mediators*

In an attempt to explain the atropine-resistant vasodilatation, which was not accompanied by kinin release, alternative mediators were considered by parallel assays on the rat stomach strip, chick rectum and rat colon. In 14 out of the 17 experiments chorda lingual nerve stimulation led to the following responses: the rat stomach strip relaxed or relaxed and contracted. The response usually persisted after atropine, but often only relaxation developed. The responses of the chick rectum were inconsistent. The rat colon consistently relaxed, although a small contraction was occasionally seen afterwards. The response was usually unaffected by atropine (Figure 6). This relaxation excluded prostaglandins as mediators of the chorda vasodilatation since they contract the colon. Stimulation of the vagosympathetic trunk and massage of the gland also did not lead to tissue responses typical of prostaglandins. Furthermore, the administration of aspirin (100 mg/kg i.v.) to 2 dogs and indomethacin (1–2 mg/kg i.v.) to 3

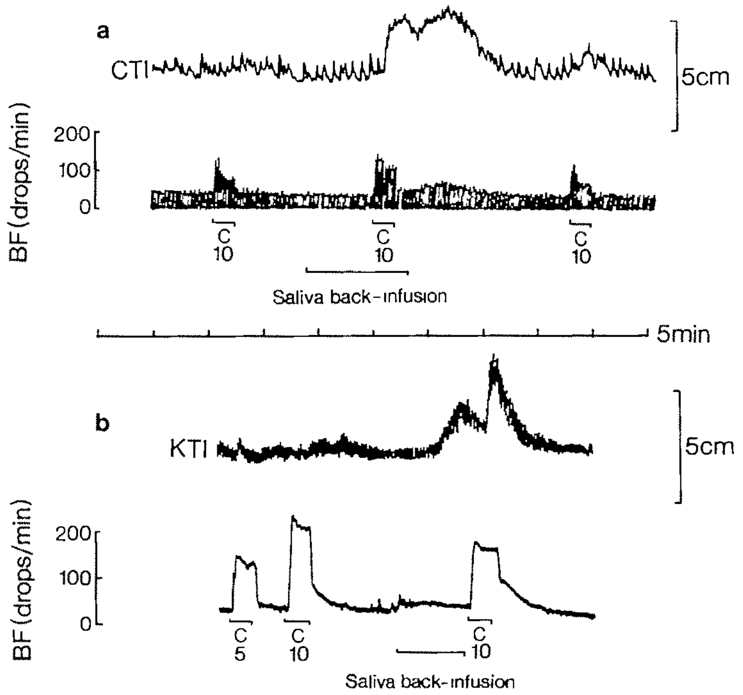


**Figure 5** Blood flow (BF) and appearance of kinin on chorda lingual nerve stimulation at frequencies of 5 or 10 Hz (C5, C10) after infusion of saliva 0.1 ml/min retrogradely into the submandibular duct in dogs treated with atropine (1 mg/kg i.v.). (a) CTI, contractions of cat terminal ileum. (b) KTI, contractions of kitten terminal ileum. Note that in (a) kinin only appears following stimulation of the nerve, whereas in (b) there is an increase in basal blood flow and appearance of kinin before chorda stimulation.

dogs had no effect on the responses to parasympathetic nerve stimulation.

The responses of the isolated tissues induced by chorda stimulation or by bradykinin either before or after atropine were not due to a higher $P\text{O}_2$ in the superfusate; in 3 experiments tissues superfused with blood from the femoral artery produced a different pattern of responses (Figure 6).

## Discussion

Perhaps the most intriguing feature of this investigation was the evidence that kallikrein or kinin was released into the venous outflow from the submandibular gland during chorda lingual nerve stimulation before atropine; yet, kallikrein did not appear to be responsible for the vasodilatation.

Stimulation of the chorda led to contractions of the cat assay tissues, and their enhancement by BPP suggested that stimulation of the chorda lingual nerve before atropine, could induce the release of bradykinin or kallikrein into the venous effluent. If kallikrein were released from the gland, the kinin detected would be generated in the venous blood between the gland and the assay tissues. In the perfusion experiments of Hilton & Lewis (1956) kallikrein and not kinin was detected after chorda stimulation. The present experiments do not distinguish which of the two factors was released.

Several factors argue against a role for kinins in mediating the vasodilator response to chorda stimulation. Increasing the interstitial kallikrein concentration by back infusion of saliva or by chorda stimulation with the excretory duct clamped, did not lead to significantly increased vasodilator responses. In some animals, repeated chorda stimulation led to a diminishing kinin output without a corresponding diminution in the vasodilator response. Following atropine, the output of kinin always declined to undetectable levels while the vasodilatation persisted. The contractions of the cat assay tissues seen in six experiments after atropine were not potentiated by BPP, and therefore not due to kinin. It seems unlikely that the amount of kinin required to cause vasodilatation was below the threshold of our assay system

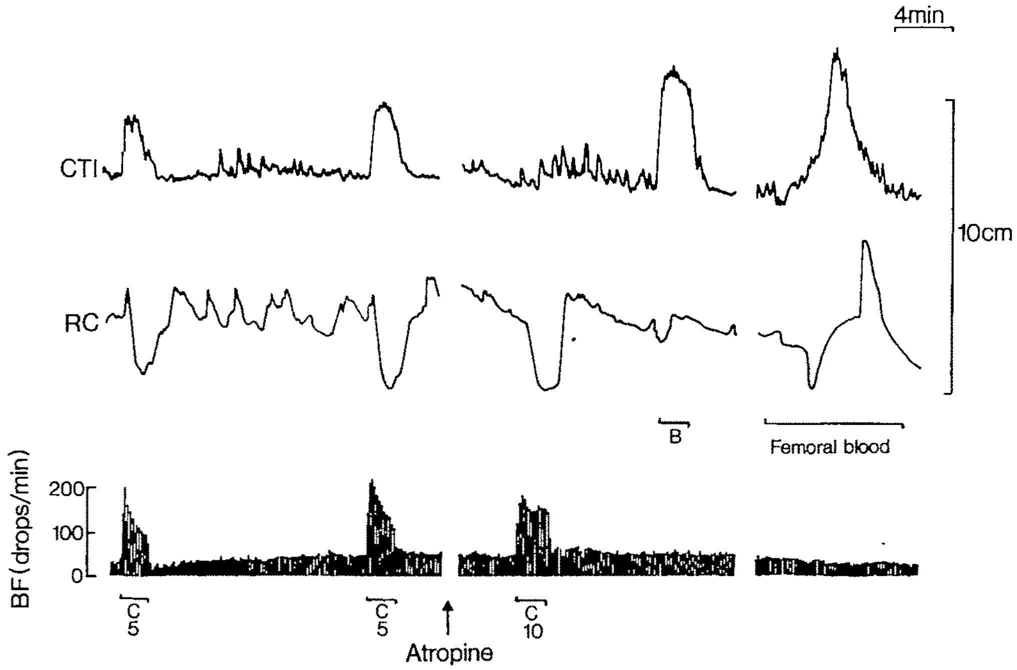


**Figure 6** Chorda lingual nerve stimulation and appearance of rat colon (RC) relaxing and cat terminal ileum (CTI) contracting material; C5, C10 = chorda lingual nerve stimulation at 5 or 10 Hz. After atropine the contractile response of the CTI disappeared although the tissue remained sensitive to bradykinin (B 4 ng/ml), whereas relaxation of the rat colon persisted. During the break in the record after atropine, there were six periods of chorda stimulation. Superfusion with femoral arterial blood induced a different pattern of responses from that caused by chorda stimulation.

because the relationship between stimulus frequency and kinin-output (Figure 2) was such that the vasodilator responses seen after atropine would be associated with detectable amounts of kinin. There was no change in tissue response to kinin as atropine did not reduce the sensitivity to injected bradykinin. Furthermore, $BPP_{9a}$ given intra-arterially failed to enhance the vasodilator response to chorda stimulation although it did increase the contractions of the cat tissues and the vasodilatation produced by bradykinin. Similar observations were made in the cat by Schachter, Barton & Karpinski (1973), who also used $BPP_{9a}$, and by Smaje (1967) who employed mercaptoethanol in the rabbit to inhibit the kinin-destroying enzymes. Evidence for an atropine-resistant vasodilatation in glands perfused with media lacking substrate for kallikrein also casts doubt on claims that kinin mediates the vasodilatation (Bhoola *et al.*, 1965; Gautvik, 1970a; Poulsen, 1975).

If kinin does not produce the vasodilatation, could vasodilatation partly determine the amount of kinin released into the venous effluent? This could explain the relationship between stimulus frequency and kinin release (Figure 2) and the parallel increase in kinin output and vasodilatation following the first stimulus after atropine. Increased glandular blood flow could have increased the washout rate of the interstitial space, possibly by increased convective mixing in the interstitium (Mellander & Lundgren, 1968), thus carrying more kallikrein into the venous effluent. However, as atropine blocks the release of kallikrein from the gland successive chorda stimulations lead to less and less kinin in the venous output due to exhaustion of the kallikrein remaining in the ducts.

A reduction in kallikrein output after atropine had been noted by Hilton & Lewis (1956) but reduction to zero was not seen. This might have been because these authors apparently included in their collection glandular perfusate from the first stimulus after atropine. Atropine also prevents the consumption of kininogen by salivary kallikrein, which is specifically increased by chorda stimulation (Gautvik, Hilton & Torres, 1970). The inference from these experiments and those of the present paper is that the release of kallikrein by chorda stimulation, like the rest of the components of saliva, is prevented by atropine. Thus kallikrein can be released by chorda stimulation and can contribute to the accompanying functional vasodilatation, but it is not the main mediator and it is not involved in the vasodilator response remaining after atropine.

In the present experiments the blood bathing the assay tissues came from two sources; the gland and the external jugular vein. During vasodilatation the proportion of gland blood increased and this could have contributed to the observed effects. However, chorda stimulation induced kinin release, before but not after atropine, whereas the blood flow increase was similar in both instances (Figures 1, 2 and 4). This implies that the superfusate contained similar proportions of blood to the gland and that the lack of kinin release after atropine was not an artefact.

The vasodilator substance released from the atropine-treated gland was not a prostaglandin as the responses of the assay tissues to prostaglandins differed from those induced by chorda stimulation. Also the effects of chorda stimulation were not affected by aspirin and indomethacin which block prostaglandin synthesis (Vane, 1971; Ferreira, Moncada & Vane, 1971). Histamine and potassium ions have been eliminated by previous investigations (Hilton & Lewis, 1955a, Darke & Smaje, 1972). Preliminary experiments showed that after atropine the vasodilator adenosine produced a relaxation of the isolated tissues similar to that found on chorda stimulation.

Since no single agent seems to mediate functional vasodilatation in the submandibular gland, could a combination of substances be responsible? Gautvik (1970a,b) suggested that the vasodilator response consists of an early phase due to activation of atropine-resistant parasympathetic vasodilator nerves and a phase in which kallikrein maintains the vasodilatation. Darke & Smaje (1972) also demonstrated a two-phase vasodilatation; atropine affected the first phase more than the second, and in glands depleted of kallikrein parasympathetic vasodilatation was smaller than in controls (Darke & Smaje, 1973). The available information suggests that kallikrein is not the sole mediator of vasodilatation in the submandibular gland but it may modulate a vasodilatation produced by another agent whose nature and mode of action remains unknown.

We are grateful to Dr J.R. Vane for his support during this work, and for his helpful criticism of the manuscript.

## References

BEILENSON, SUSANNE, SCHACHTER, M. & SMAJE, L.H. (1968). Secretion of kallikrein and its role in vasodilatation in the submaxillary gland. *J. Physiol. Lond.*, **199**, 303–317.

BHOOLA, K.D., MORLEY, J., SCHACHTER, M. & SMAJE, L.H. (1965). Vasodilatation in the submaxillary gland of the cat. *J. Physiol. Lond.*, **179**, 172–184.

DARKE, A.C. & SMAJE, L.H. (1972). Dependence of functional vasodilatation in the cat submaxillary gland upon stimulation frequency. *J. Physiol. Lond.*, **226**, 191–203.

DARKE, A.C. & SMAJE, L.H. (1973). The effect of chronic duct ligation on the vascular and secretory responses of the cat's submaxillary gland. *J. Physiol. Lond.*, **228**, 361–376.

FERREIRA, S.H. (1965). A bradykinin potentiating factor (BPF) present in the venom of *Bothrops jararaca*. *Br. J. Pharmac.*, **24**, 163–169.

FERREIRA, S.H., CORRADO, A.P. & ROCHA E SILVA, M. (1969). Kinins and vasodilatation of the submandibular salivary glands of dogs. *Abstract of the Fourth International Congress on Pharmacology* pp. 208–209.

FERREIRA, S.H., MONCADA, S. & VANE, J.R. (1971). Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature, New Biol.*, **231**, 237–239.

FERREIRA, S.H., NG, K.K. & VANE, J.R. (1973). The continuous bioassay of the release and disappearance of histamine in the circulation. *Br. J. Pharmac.*, **49**, 543–553.

FERREIRA, S.H. & VANE, J.R. (1967a). The detection and estimation of bradykinin in the circulating blood. *Br. J. Pharmac.*, **29**, 367–377.

FERREIRA, S.H. & VANE, J.R. (1967b). The disappearance of bradykinin and eledoisin in the circulation and vascular beds of the cat. *Br. J. Pharmac.*, **30**, 417–424.

GAUTVIK, K. (1970a). The interaction of two different vasodilator mechanisms in the chorda-tympani activated submandibular salivary gland. *Acta physiol. scand.*, **79**, 188–203.

GAUTVIK, K. (1970b). Parasympathetic neuro-effector transmission and functional vasodilatation in the submandibular salivary gland of cats. *Acta physiol. scand.*, **79**, 204-215.

GAUTVIK, K.M., HILTON, S.M. & TORRES, SONIA H. (1970). Consumption of kininogen in the submandibular salivary gland when activated by chorda stimulation. *J. Physiol. Lond.*, **211**, 49–61.

GILLMORE, N., VANE, J.R. & WYLLIE, J.H. (1968). Prostaglandins released by the spleen. *Nature, Lond.*, **218**, 1135–1140.

GREENE, L.J., CAMARGO, A.C.M., KREIGER, E.M., STEWART, J.M. & FERREIRA, S.H. (1972). Inhibition of the conversion of angiotensin I to II and potentiation of bradykinin by small peptides present in *Bothrops jararaca* venom. *Circulation Res.*, **31**, Suppl. II 62–71.

HEIDENHAIN, R. (1872). Über die Wirkung einiger Gifte auf die Nerven der glandula submaxillaris. *Pflügers Arch. ges. Physiol.*, **5**, 309–318.

HILTON, S.M. & LEWIS, G.P. (1955a). The cause of the vasodilatation accompanying activity in the submandibular salivary gland. *J. Physiol. Lond.*, **128**, 253–248.

HILTON, S.M. & LEWIS, G.P. (1955b). The mechanism of the functional hyperaemia in the submandibular salivary gland. *J. Physiol. Lond.*, **129**, 253–271.

HILTON, S.M. & LEWIS, G.P. (1956). The relationship between glandular activity, bradykinin formation and functional vasodilatation in the submandibular salivary gland. *J. Physiol. Lond.*, **134**, 471–483.

MANN, MONICA & WEST, G.B. (1950). The nature of hepatic and splenic sympathin. *Br. J. Pharmac. Chemother.*, **5**, 173–177.

MELLANDER, S. & LUNDGREN, O. (1968). Augmentation of tissue clearance by transcapillary fluid movement. In *Circulation of Skeletal Muscle.* ed. Hudlicka, O., pp. 93–98, Oxford: Pergamon.

NOBILI, M.B. (1965). Sulla inattivazione della eledoisina e della physalaemin da parte del sangue totale e di omogenati tessutali di alcuni vertebrati. *Archs int. Pharmacodyn. Thér.*, **158**, 187–201.

POULSEN, J.H. (1975). Two phases of chorda-lingual induced vasodilatation in the cat's submandibular gland during prolonged perfusion with Locke solution. *J. Physiol. Lond.*, **253**, 79–94.

REGOLI, D. & VANE, J.R. (1964). A sensitive method for the assay of angiotensin. *Br. J. Pharmac. Chemother.*, **23**, 351–359.

SCHACHTER, M., BARTON, SUSANNE & KARPINSKI, E. (1973). Analysis of vasodilatation in the submaxillary gland using potentiators of acetylcholine and kinins. *Experientia*, **29**, 973–974.

SCHACHTER, M. & BEILENSON, SUSANNE (1967). Kallikrein and vasodilatation in the submaxillary gland. *Gastroenterology*, **52**, 401–405.

SKINNER, N.S. Jr & WEBSTER, MARION E. (1968). Kinins, β-adrenergic receptors and functional vasodilatation in the submaxillary gland of the cat. *J. Physiol. Lond.*, **195**, 505–519.

SMAJE, L.H. (1967). Regulation of blood flow in the salivary gland. Ph.D. Thesis, University of London.

UNGAR, G. & PARROT, J.L. (1936). Sur la présence de la callicréine dans la salive, et la possibilité de son intervention dans la transmission chimique de l'influx nerveux. *C.r. Seanc. Soc. Biol.*, **122**, 1052–1055.

VANE, J.R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Br. J. Pharmac. Chemother.*, **12**, 344–349.

VANE, J.R. (1964). The use of isolated organs for detecting active substances in the circulating blood. *Br. J. Pharmac. Chemother.*, **23**, 360–373.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature, New Biol.*, **231**, 232–235.

*(Received January 9, 1976.*
*Revised April 22, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 211–221

# RECEPTORS FOR 5-HYDROXYTRYPTAMINE AND NORADRENALINE IN RABBIT ISOLATED EAR ARTERY AND AORTA

EIRA APPERLEY, P.P.A. HUMPHREY & G.P. LEVY
Department of Pharmacology, Allen & Hanburys Research Limited, Ware, Hertfordshire SG12 0DJ

**1** 5-Hydroxytryptamine (5-HT) is thought to be implicated in the vascular disturbances of the external carotid artery bed associated with migraine. As part of a study of the pharmacology of some 5-HT antagonists used in the treatment of migraine we have examined the interactions of these drugs with 5-HT and noradrenaline in rabbit isolated ear artery and aortic strip. The results provide new information on the distribution of 5-HT-receptors and $\alpha$-receptors in these preparations.

**2** In the aorta, 5-HT and noradrenaline were of similar potency in producing contractions. Methysergide produced very small contractions and was about 1000 times less potent than the other two agonists.

**3** In the ear artery noradrenaline produced monophasic vasoconstrictor responses, whereas 5-HT and methysergide produced prolonged biphasic responses. 5-HT was about 700 times less potent and methysergide about 4500 times less potent than noradrenaline. Methysergide was a better agonist in the ear artery than in the aorta. Biphasic responses to 5-HT and methysergide were also obtained in ear arteries from reserpine-treated rabbits indicating that neither agonist was acting by releasing endogenous noradrenaline.

**4** Pizotifen, cyproheptadine and phentolamine had no agonistic actions in either the aorta or ear artery.

**5** In the aorta methysergide, pizotifen and cyproheptadine were potent antagonists of 5-HT and much weaker antagonists of noradrenaline. Phentolamine possessed the opposite profile of selectivity. These results show that there are distinct receptors for 5-HT and noradrenaline in rabbit aorta.

**6** In the ear artery the $pA_2$ values for each of the four antagonists were virtually identical against 5-HT and noradrenaline and similar to those obtained on $\alpha$-adrenoceptors in the aorta. We conclude that 5-HT and noradrenaline act directly at $\alpha$-receptors to produce vasoconstriction in the ear artery and that this preparation does not contain specific 5-HT-receptors.

**7** This insight into the distribution of 5-HT-receptors and $\alpha$-receptors allows interpretation of the various actions of methysergide. In the aorta, methysergide was a potent antagonist at 5-HT-receptors and a weak partial agonist at $\alpha$-receptors. In the ear artery, methysergide was a partial agonist at $\alpha$-receptors; it was only a weak antagonist of 5-HT because this preparation does not contain specific 5-HT-receptors.

**8** The cross-reactivity demonstrated throughout these experiments indicates that 5-HT-receptors and $\alpha$-receptors, although distinct entities, have features in common.

**9** These results are discussed in relation to the mode of action of methysergide, pizotifen and cyproheptadine in the treatment of migraine.

## Introduction

Although the aetiology of migraine is not clear there is considerable evidence implicating 5-hydroxytryptamine (5-HT) in the vascular disturbances of the external carotid artery bed associated with the syndrome (see Lance, Anthony & Hinterberger, 1970). Methysergide, a proven migraine prophylactic drug, is well known as a 5-HT antagonist (see Fozard, 1975 for references). However, recent studies reveal that methysergide also has an agonistic vasoconstrictor action in the carotid vasculature (Fozard, 1973; Saxena, 1974; Apperley, Humphrey & Levy, 1974). As part of a wider study into the mechanisms of action of drugs used in the treatment of migraine we have analysed the agonistic and antagonistic actions of methysergide at the receptor level. Two other drugs used in the treatment of migraine and known to interact with 5-HT, namely pizotifen and cyproheptadine, have also been examined. It was hoped that a better understanding of the actions of these drugs at the receptor level would help to explain their therapeutic effects and also provide clues about the aetiology of the syndrome. Experiments have been carried out with the rabbit central ear artery, a branch of the external carotid artery, and for comparison, the aorta. The results show that methysergide, pizotifen and cyproheptadine can act on both 5-HT and noradrenaline receptors and provide new insight into the distribution of these receptors in the two preparations.

Some of these results were presented to a meeting of the British Pharmacological Society (Apperley, Humphrey & Levy, 1974).

## Methods

New Zealand white rabbits of either sex, body weight 2–3 kg, were used.

### *Rabbit aortic strip*

Thoracic aortic arteries were removed from animals killed with pentobarbitone (400 mg i.v.). The aortae were cut spirally into strips as described by Furchgott & Bhadrakom (1953), four preparations being obtained from each animal. Each strip was suspended in a 20 ml organ bath containing a modified Krebs solution. Tension was measured with a Statham Microscale Accessory (Model UL5) attached to a Statham Universal Transducing Cell (Model UC3). Strips were approximately 2 cm in length at an applied resting tension of 400–500 mg.

Preparations were left for 3 h before the start of the experiment. During this period three doses of potassium chloride ($3.0 \times 10^{-2}$ mol/l) were administered at intervals of 45–60 minutes. The resting tension decreased slightly and the size of the contractile response to potassium chloride increased to an almost constant level.

A single agonist (noradrenaline, 5-HT, methysergide or potassium chloride) was administered cumulatively to each strip. Increase in tension, expressed as % maximum response, was plotted against agonist concentration on a log scale. The potency of each agonist was calculated from its concentration-effect curve. The agonist was then washed from the bath and the potency of an antagonist (phentolamine, methysergide, pizotifen or cyproheptadine) determined in the same preparations. Graded concentrations of antagonist were added to three of the strips whilst the fourth strip acted as a control. After 30 min, concentration-effect curves to the agonist were re-determined in all four strips. The degree of antagonism was quantified in terms of the dose-ratio, which was determined by the ratio of the $EC_{50}$ values before and after the addition of antagonist. Correction was made for any spontaneous change in sensitivity by dividing the dose-ratios obtained in the strips exposed to antagonist by the dose-ratio obtained in the control strip. The sensitivity of the control strips to the various agonists decreased during the experiments by about 2-fold. The results were plotted graphically in the form of a Schild plot as log (agonist dose-ratio −1) on log antagonist concentration (mol/l) (Arunlakshana & Schild, 1959). The slope of the regression and the $pA_2$ value were estimated.

### *Rabbit isolated ear artery*

Right and left auricular arteries were removed from animals anaesthetized with pentobarbitone (36 mg/kg i.v.). The vessels were carefully cleared of all connective tissue and perfused intraluminally with modified Krebs solution at a constant flow rate of 7 ml/min by means of a peristaltic pump (H.R. Flow Inducer, Watson-Marlow Ltd.). The distal end of the artery was not cannulated, so that the perfusion fluid, after contact with the intraluminal surface, bathed the extraluminal surface before being removed by overflow. The bath volume was thereby kept at a constant volume of 15 ml. Changes in intraluminal pressure were recorded from a pressure transducer (Bell & Howell Ltd., type 4–422–0001) attached to a side arm of the perfusion cannula. Bolus injections of the agonists were made into the perfusion fluid immediately before its passage through the peristaltic pump. A constant injection volume of 100 µl which produced no injection artefact was used. This procedure is similar to that described by de la Lande & Rand (1965).

Paired preparations from the same animal were used in parallel experiments. The arteries were perfused for 60 min before the experiment started. A single agonist was then administered at 5 or 6 dose-levels to the two arteries, with a 5–10 min interval between doses. Increase in perfusion pressure was plotted against dose on a log scale. The potency of each agonist was estimated from the dose-effect curves. The potency of an antagonist was then determined in the same two preparations. One artery acted as a control and was perfused with Krebs solution alone. The second artery was perfused for 30 min with Krebs solution containing the test antagonist. Agonist dose-effect curves were then re-determined in test and control arteries. This procedure was repeated twice more using successively higher concentrations of antagonist. Since maximum responses were rarely obtained and individual dose-response curves varied slightly in shape, dose-ratios were estimated at arbitrarily determined response levels on the linear portion of the curves. Dose-ratios were corrected for spontaneous changes in sensitivity. The sensitivity to vasopressin decreased during the experiment by about 2-fold; sensitivity to noradrenaline, 5-HT or methysergide usually increased, the maximum increase being 5-fold. The results were analysed by the Schild plot as described under rabbit aortic strip.

### *Pretreatment with reserpine*

Reserpine was dissolved in distilled water containing 20% w/v ascorbic acid. Rabbits were injected with reserpine (1 mg/kg i.p.) 18 h before removal of the arteries. This procedure has been shown to eliminate the indirect action of tyramine, which results from release of endogenous noradrenaline (Farmer, 1966).

### *Physiological saline*

A modified Krebs solution with the following composition (mmol/l) was used: $Na^+$ 143.4, $K^+$ 5.9, $Mg^{++}$ 0.6, $Ca^{++}$ 1.3, $Cl^-$ 124.5, $H_2PO_4^-$ 1.2, $SO_4^{++}$ 0.6, $HCO_3^-$ 25.0, glucose 11.1, gassed with 95% $O_2$ and 5% $CO_2$ at 37°C.

### *Drugs*

The following drugs were used: (−)-noradrenaline bitartrate, mol. wt. 337.3 (Koch-Light), 5-hydroxytryptamine creatinine sulphate, mol. wt. 405.4 (Koch-Light), methysergide bimaleate, mol. wt. 469.5 (Sandoz), pizotifen hydrogen maleate, mol. wt. 295.4 (Sandoz), phentolamine methane sulphonate, mol. wt. 337.5 (Ciba), cyproheptadine hydrochloride, mol. wt. 350.9 (Merck Sharp & Dohme) and lysine-vasopressin mol. wt. 1056.3 (Sigma). 5-HT, methysergide, pizotifen and cyproheptadine were dissolved in distilled water, and phentolamine and vasopressin in 0.9% w/v NaCl solution (saline). Noradrenaline was dissolved in saline containing ascorbic acid (0.2 mg/ml).

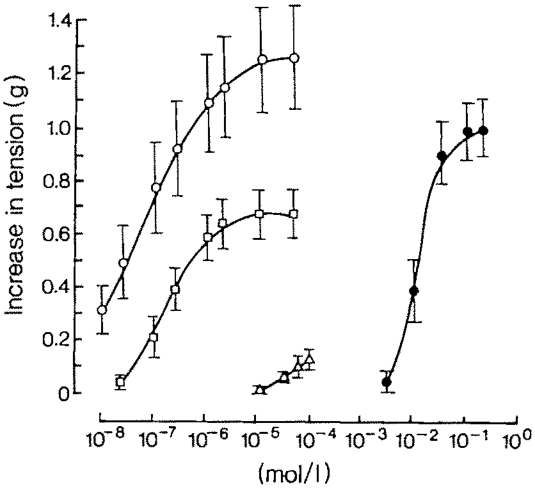


**Figure 1** Rabbit aortic strip. Concentration-effect curves for noradrenaline (○), 5-hydroxytryptamine (□), methysergide (△) and potassium chloride (●). Each point is the mean response from 8 strips, each taken from a different animal. Vertical lines show s.e. mean. Note the very weak agonistic action of methysergide.

## Results

### *Rabbit aortic strip*

*Agonists* Potassium chloride produced contractions of the aorta in the concentration-range $3.0\times10^{-3}$–$2.0\times10^{-1}$ mol/l with a maximum tension of $1.0\pm0.1$ g (mean ± s.e. mean, $n=8$) (Figure 1). Noradrenaline and 5-HT produced contractions over similar concentration ranges, $1.0\times10^{-8}$–$5.0\times10^{-5}$ mol/l, with a maximum tension of $1.3\pm0.2$ g ($n=8$) and $0.7\pm0.1$($n=8$) respectively. 5-HT ($EC_{50}$ $1.7\times10^{-7}$ mol/l) was about 4 times less active than noradrenaline ($EC_{50}$ $4.3\times10^{-8}$ mol/l) (Figure 1).

**Table 1** Effect of pretreatment with high concentrations of noradrenaline or 5-hydroxytryptamine on contractile responses to methysergide or histamine in rabbit aortic strip

| *Pretreatment* | *Agonist* | *Maximal response as % of the maximal response in strips pretreated with potassium chloride ($3.0\times10^{-2}$ mol/l)* |
|---|---|---|
| Noradrenaline<br>($5.0\times10^{-5}$ mol/l) | Methysergide | 19 (0–34) |
| 5-Hydroxytryptamine<br>($5.0\times10^{-6}$ mol/l) | Methysergide | 101 (49–198) |
| Noradrenaline<br>($5.0\times10^{-5}$ mol/l) | Histamine | 87 (60–121) |

Each value is the mean of 4–8 determinations (range).

Methysergide was a weak agonist over the concentration-range $1.0 \times 10^{-5}$–$1.0 \times 10^{-4}$ mol/l (Figure 1), the response at the highest concentration being $0.14 \pm 0.04$ g ($n=8$), which is only 11% of the maximum contraction produced by noradrenaline. Comparison of potencies at this level shows that methysergide is over 1000 times less potent than noradrenaline or 5-HT. Concentrations of methysergide higher than $1.0 \times 10^{-4}$ mol/l were not tested because this was the practical limit of its solubility.

During the course of these experiments we noticed that methysergide was even less active in preparations

**Table 2** Interactions between agonists and antagonists in rabbit aortic strip

| | | *Agonist* | |
|---|---|---|---|
| *Antagonist* | | *Noradrenaline* | *5-hydroxytryptamine* |
| Phentolamine | $pA_2$ | 7.96 | 6.21 |
| | | (7.76–8.16) | (5.52–6.90) |
| | Slope | 0.91 | 0.93 |
| | | (0.79–1.03) | (0.60–1.26) |
| Pizotifen | $pA_2$ | 6.69 | 9.42 |
| | | (6.23–7.15) | (8.18–10.66) |
| | Slope | 0.78 | 1.01 |
| | | (0.53–1.03) | (0.29–1.73) |
| Cyproheptadine | $pA_2$ | 6.69 | 8.73 |
| | | (6.05–7.33) | (8.36–9.10) |
| | Slope | 1.02 | 0.90 |
| | | (0.69–1.35) | (0.71–1.09) |
| Methysergide | $pA_2$ | 5.29 | 8.49 |
| | | (4.95–5.63) | (7.85–9.14) |
| | Slope | 1.20 | 0.78 |
| | | (0.74–1.66) | (0.51–1.05) |

Each value is the mean (95% confidence limits) of 4–8 separate estimates. Tissues exposed to antagonists for 30 min before agonist concentration-effect curves re-determined.

**Table 3** Comparison of the specific and non-specific blocking actions of phentolamine, pizotifen, cyproheptadine and methysergide in rabbit aortic strip

| | | *Agonist dose-ratio at antagonist concentration (mol/l) of* | | | |
|---|---|---|---|---|---|
| *Antagonist* | *Agonist* | $1.0 \times 10^{-6}$ | $5.0 \times 10^{-6}$ | $1.0 \times 10^{-5}$ | $1.0 \times 10^{-4}$ |
| Phentolamine | 5-Hydroxytryptamine | — | — | 11 | 92 |
| | | | | (6–22) | (56–151) |
| | Potassium chloride | — | — | 1.2 | 3.7 |
| | | | | (0.8–1.8) | (2.6–5.5) |
| Pizotifen | Noradrenaline | 4.0 | 25 | 50 | — |
| | | (3.0–5.3) | (20–31) | (32–79) | |
| | Potassium chloride | 0.9 | 2.9 | 4.9 | — |
| | | (0.8–1.1) | (2.5–3.3) | (3.5–6.6) | |
| Cyproheptadine | Noradrenaline | 7.4 | 42 | 96 | — |
| | | (6.7–8.0) | (25–71) | (64–145) | |
| | Potassium chloride | 2.4 | 5.2 | 5.0 | — |
| | | (1.8–3.1) | (3.7–7.3) | (3.4–7.2) | |
| Methysergide | Noradrenaline | — | — | 2.6 | 22 |
| | | | | (1.7–3.9) | (14–33) |
| | Potassium chloride | — | — | 0.7 | 0.9 |
| | | | | (0.5–1.0) | (0.5–1.6) |

Each value is the mean of 4–8 determinations (95% confidence limits). Tissues exposed to antagonists for 30 min before agonist concentration-effect curves re-determined. For each antagonist the potassium chloride blocking action is compared with the weaker of the two amine blocking actions.

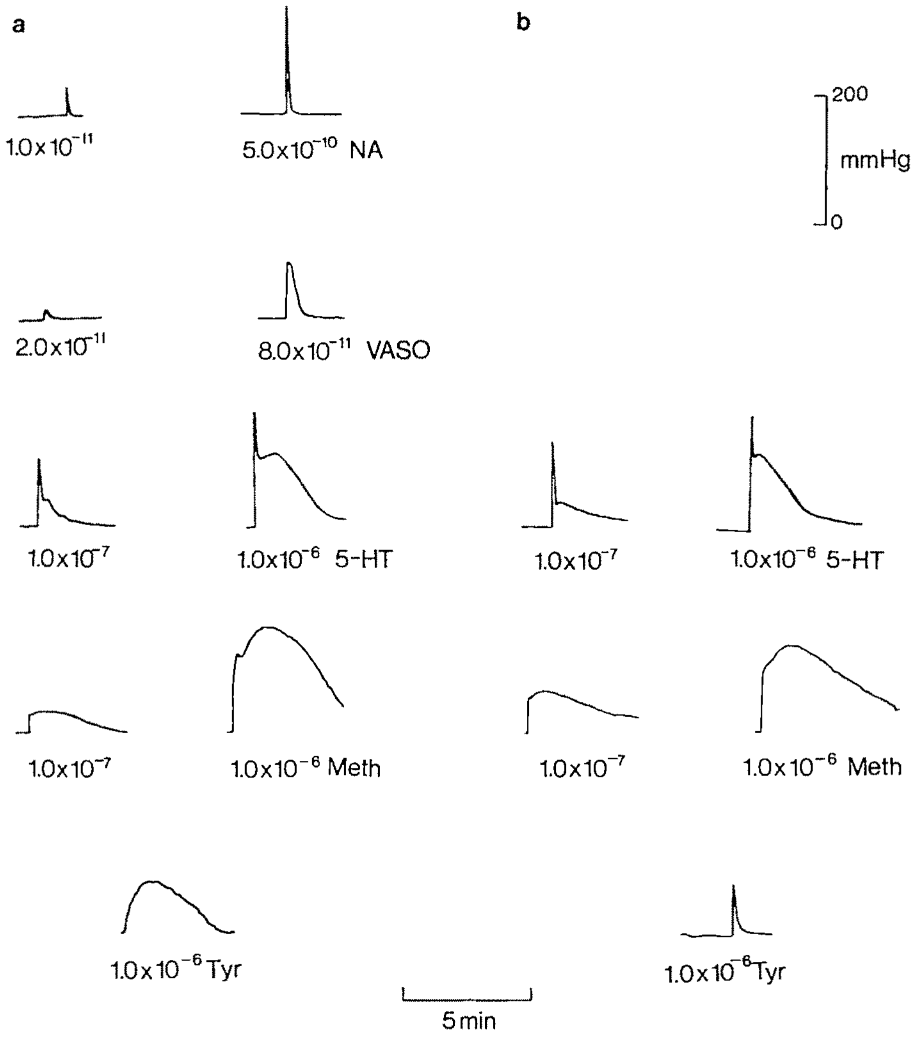


**Figure 2** Rabbit isolated ear artery. Vasoconstrictor responses to bolus injections of noradrenaline (NA), vasopressin (VASO), 5-hydroxytryptamine (5-HT), methysergide (Meth) and tyramine (Tyr) (a) in normal arteries and (b) in arteries removed from reserpine-treated rabbits. Doses are expressed in mol. Note that reserpine-treatment converts the prolonged response to tyramine to a transient response but has no effect on the prolonged responses to 5-HT and methysergide.

previously exposed to noradrenaline and the phenomenon was further investigated. In each experiment two aortic strips were exposed to either noradrenaline ($5.0\times10^{-5}$ mol/l) or 5-HT ($5.0\times10^{-6}$ mol/l) and the other two strips to potassium chloride ($3.0\times10^{-2}$ mol/l), the concentrations of each agonist being selected to produce a virtually maximal response. The agonists were washed out after 10 min and the strips left for 90 min before concentration-effect curves to either methysergide ($1.0\times10^{-5}$–$1.0\times10^{-4}$ mol/l) or histamine ($1.0\times10^{-8}$–$1.0\times10^{-4}$ mol/l) were determined. The results are summarized in Table 1. Prior exposure to noradrenaline, but not to 5-HT, greatly reduced the maximal response to methysergide. In contrast, the maximal response to histamine was unaffected by prior exposure to noradrenaline.

Phentolamine ($1.0\times10^{-8}$–$1.0\times10^{-4}$ mol/l), pizotifen ($1.0\times10^{-8}$–$1.0\times10^{-4}$ mol/l), cyproheptadine ($1.0\times10^{-8}$–$1.0\times10^{-4}$ mol/l) and vasopressin ($0.8\times10^{-10}$–$0.8\times10^{-7}$ mol/l) were not agonists.

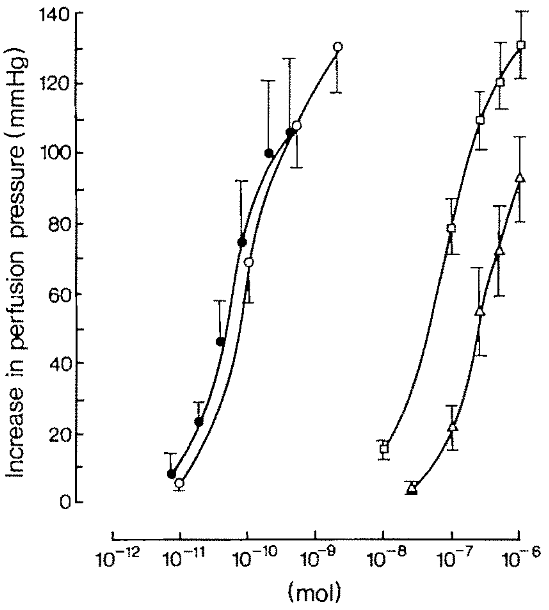


**Figure 3** Rabbit isolated ear artery. Dose-effect curves for vasopressin (●), noradrenaline (○), 5-hydroxytryptamine (□) and methysergide (△). Each point is the mean response from 16 arteries obtained from eight animals. Vertical lines show s.e. mean. Note that 5-HT and methysergide are considerably less potent than noradrenaline.

*Antagonists* Methysergide, pizotifen, cyproheptadine and phentolamine were investigated as antagonists of 5-HT and noradrenaline. The results are summarized in Table 2. Each caused parallel concentration-dependent shifts to the right in the 5-HT and noradrenaline concentration-effect curves. The Schild plots were linear with slopes not significantly different from unity. These findings suggest competitive antagonism in each case.

For each antagonist there was a clear difference in potency against 5-HT and noradrenaline (Table 2). Methysergide, pizotifen and cyproheptadine were potent 5-HT antagonists and much weaker noradrenaline antagonists. Phentolamine had the opposite profile of selectivity.

The effect of these antagonists on contractile responses to potassium chloride was also examined in order to distinguish between specific and any non-specific blocking activity. The results, summarized in Table 3, show that over the concentration ranges tested these antagonists possess little non-specific blocking activity.

The effects of antagonists on the small contractile responses to methysergide ($1.0\times10^{-5}$–$1.0\times10^{-4}$ mol/l) were also investigated. These responses were abolished by phentolamine at a concentration ($1.0\times10^{-6}$ mol/l) which antagonized noradrenaline with little or no effect on 5-HT, but were only slightly antagonized (methysergide dose-ratio of 2 to 3, $n=4$) by pizotifen at a concentration ($1.0\times10^{-7}$ mol/l) which produced a dose-ratio of about 400 against 5-HT.

### *Rabbit isolated ear artery*

*Agonists* Individual vasoconstrictor responses are shown in Figure 2 and dose-response curves in Figure 3.

Vasopressin ($8.0\times10^{-12}$–$4.0\times10^{-10}$ mol) produced prolonged responses, usually monophasic but occasionally biphasic, with a maximal response of $106\pm21$ mmHg (mean ± s.e. mean, $n=6$).

Noradrenaline ($1.0\times10^{-11}$–$2.0\times10^{-9}$ mol) produced transient monophasic responses with a maximal or near-maximal response of $130\pm13$ mmHg ($n=16$).

Responses to 5-HT ($1.0\times10^{-8}$–$1.0\times10^{-6}$ mol) were prolonged and biphasic, with the initial phase consistently larger but of shorter duration than the second phase. Results have been presented only for the initial phase. 5-HT produced a near-maximal response of $131\pm9$ mmHg ($n=16$) at $1.0\times10^{-6}$ mol; it was about 700 times less potent than noradrenaline, in contrast to the finding in the aorta where these two agonists were similar in potency.

Methysergide was a better agonist in the ear artery than in the aorta. The responses were prolonged and often two phases could be distinguished, an initial phase of short duration and a prolonged and slightly larger second phase. Results have been presented only for the second phase. At $1.0\times10^{-6}$ mol, the highest dose that could be tested, methysergide produced a response of $93\pm12$ mmHg ($n=16$). Methysergide was about 6 times less potent than 5-HT and about 4500 times less potent than noradrenaline. The sensitivity of the ear artery to methysergide was not reduced by prior exposure to noradrenaline, in contrast to our findings in the aorta.

In order to determine the concentration-range over which methysergide was an agonist, a second series of experiments was carried out in which the drug was perfused intraluminally in graded concentrations instead of being given by bolus injection. In four arteries, threshold vasoconstriction (5% of the maximum response to noradrenaline) occurred at $1.0\times10^{-6}$ mol/l and maximum vasoconstriction (76% of the maximum response to noradrenaline) at $1.0\times10^{-4}$ mol/l.

Experiments were carried out in arteries from reserpine-treated rabbits to determine whether part of the response to 5-HT or methysergide was mediated through release of endogenous noradrenaline. Responses to 5-HT and methysergide were unchanged

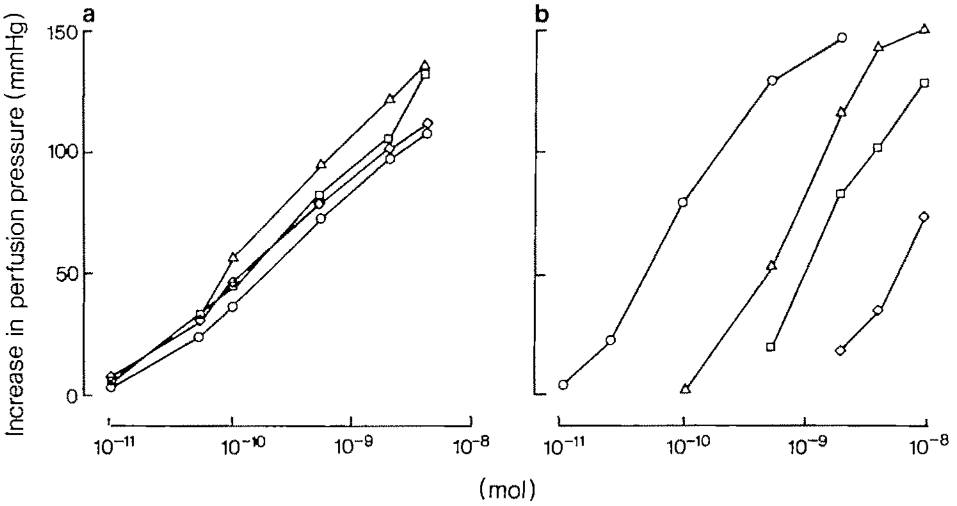


**Figure 4** Rabbit isolated ear artery. Dose-effect curves to noradrenaline (a) in a control artery perfused with Krebs alone and (b) in a test artery perfused with Krebs plus pizotifen. In each artery the first (○) second (△), third (□) and fourth (◇) dose-effect curves were determined at intervals of about 30 minutes. Pizotifen was perfused at $2.0 \times 10^{-8}$, $4.0 \times 10^{-8}$ and $1.6 \times 10^{-8}$ mol/l for 30 min prior to the second, third and fourth dose-effect curves respectively. The dose-ratios calculated for successive dose-effect curves in the control artery were 0.4, 0.7 and 0.7 and in the test artery 7.7, 18.9 and 112. When corrections were made for changes in sensitivity in the control artery the dose-ratios in the test artery became 19.9, 29.0 and 153. These values gave a Schild plot with a $pA_2$ of 6.88 and a slope of 1.03.

in these preparations (Figure 2). In contrast, the prolonged response to tyramine ($1.0 \times 10^{-6}$ mol) obtained in arteries from untreated rabbits was converted to a transient response in arteries from reserpinized rabbits (Figure 2).

Phentolamine ($1.0 \times 10^{-6}$ mol) and cyproheptadine ($1.0 \times 10^{-6}$ mol) had no vasoconstrictor activity. Pizotifen ($1.0 \times 10^{-6}$ mol) produced a transient vasoconstriction (30–45 mmHg) after the first but not after subsequent doses.

*Antagonists* Phentolamine, pizotifen and cyproheptadine antagonized vasoconstrictor responses to 5-HT, noradrenaline and methysergide. In every case, agonist dose-response curves were displaced to the right by the antagonist in a parallel, concentration-dependent manner (for example see Figure 4). Schild plots were linear with slopes not significantly different from unity. As in the aorta, these findings indicate competitive antagonism. The $pA_2$ values for each antagonist against 5-HT, noradrenaline and methysergide were virtually identical, which contrasts with the results in the aorta. These results are summarized in Table 4.

We also noted that the two phases of the response to 5-HT were antagonized equally by phentolamine or pizotifen. The $pA_2$ and slope values for phentolamine were 8.41 (7.99–8.83) and 1.02 (0.54–1.50) against the initial phase (see Table 4) and 8.34 (8.10–8.58) and 0.86 (0.62–1.11) against the second phase; the corresponding values for pizotifen were 6.79 (6.39–7.18) and 1.08 (0.77–1.39) against the initial phase (see Table 4) and 6.89 (5.70–8.08) and 1.25 (0.65–1.84) against the second phase. Similarly, the two phases of the response to methysergide, when distinguishable, were antagonized equally by phentolamine or pizotifen.

Experiments were carried out in the ear artery, as in the aorta, to determine whether the antagonists possessed any non-specific blocking activity. The results, summarized in Table 5, show that phentolamine, pizotifen and cyproheptadine had little or no effect against vasopressin-induced vasoconstriction in concentrations that strongly antagonized noradrenaline. We conclude that these antagonists possess little or no non-specific blocking action over the concentration ranges tested.

The ability of methysergide to antagonize constrictor responses to noradrenaline, 5-HT and vasopressin was also investigated. At $1.0 \times 10^{-6}$ mol/l, methysergide sensitized the ear artery to noradrenaline and vasopressin but not to 5-HT (Table 6). Higher concentrations of methysergide ($1.0 \times 10^{-5}$–$1.0 \times 10^{-4}$ mol/l) antagonized responses to noradrenaline and 5-HT but had little or no effect on those to vasopressin. The combination of sensitizing, vaso-

**Table 4** Interactions between agonists and antagonists in rabbit isolated ear artery

| *Antagonist* | | *Agonist*<br>*Noradrenaline* | <br>*5-Hydroxytryptamine* | <br>*Methysergide* |
|---|---|---|---|---|
| Phentolamine | $pA_2$ | 8.09<br>(7.64–8.64) | 8.41<br>(7.99–8.83) | 8.25<br>(7.96–8.54) |
| | Slope | 1.12<br>(0.57–1.67) | 1.02<br>(0.54–1.50) | 0.88<br>(0.75–1.02) |
| Pizotifen | $pA_2$ | 6.57<br>(6.15–6.98) | 6.79<br>(6.39–7.18) | 6.95<br>(6.48–7.42) |
| | Slope | 1.00<br>(0.76–1.24) | 1.08<br>(0.77–1.39) | 1.07<br>(0.45–1.69) |
| Cyproheptadine | $pA_2$ | 6.80<br>(6.41–7.20) | 7.02<br>(6.58–7.66) | 7.04<br>(6.59–7.49) |
| | Slope | 1.10<br>(0.51–1.70) | 0.87<br>(0.71–1.03) | 1.25<br>(0.57–1.52) |

Each value is the mean (95% confidence limits) of 4–8 separate determinations. Tissues exposed to antagonists for 30 min before agonist dose-effect curves re-determined.

**Table 5** Comparison of the specific and non-specific blocking actions of phentolamine, pizotifen and cyproheptadine in rabbit isolated ear artery

| *Antagonist* | *Agonist* | *Agonist dose-ratio at antagonist concentration (mol/l) of*<br>$1.0 \times 10^{-8}$ | <br>$1.0 \times 10^{-7}$ | <br>$1.0 \times 10^{-6}$ | <br>$4.0 \times 10^{-6}$ |
|---|---|---|---|---|---|
| Phentolamine | Noradrenaline | 2.7<br>(1.8–3.9) | 9.0<br>(4.3–19.2) | —<br>— | —<br>— |
| | Vasopressin | 1.2<br>(0.8–1.7) | 1.9<br>(1.0–3.3) | — | — |
| Pizotifen | Noradrenaline | — | — | 4.4<br>(2.1–9.3) | 19<br>(9–42) |
| | Vasopressin | — | — | 0.9<br>(0.6–1.2) | 1.1<br>(0.8–1.7) |
| Cyproheptadine | Noradrenaline | — | — | 7.7<br>(5.4–10.9) | 34<br>(13–85) |
| | Vasopressin | — | — | 2.5<br>(1.3–4.5) | 3.7<br>(2.2–6.3) |

Each value is the mean of 4–8 determinations (95% confidence limits). Tissues exposed to antagonists for 30 min before agonist dose-effect curves re-determined.

**Table 6** Effects of methysergide on responses to noradrenaline, 5-hydroxytryptamine and vasopressin in rabbit isolated ear artery

| | *Agonist dose-ratio at methysergide concentration (mol/l) of*<br>$1.0 \times 10^{-6}$ | <br>$1.0 \times 10^{-5}$ | <br>$1.0 \times 10^{-4}$ |
|---|---|---|---|
| Noradrenaline | 0.24<br>(0.15–0.36) | 3.7<br>(1.6–8.2) | 89<br>(29–279) |
| 5-Hydroxytryptamine | 1.2<br>(0.62–2.3) | 29<br>(10–83) | 343<br>(67–1758) |
| Vasopressin | 0.36<br>(0.22–0.60) | 0.86<br>(0.23–3.4) | 3.6<br>(1.3–10.1) |

Each value is the mean of 4 determinations (95% confidence limits). Dose-ratios greater than unity indicate antagonism, those less than unity indicate sensitization. Tissues exposed to methysergide for 30 min before agonist dose-effect curves re-determined.

constrictor and blocking properties present in methysergide makes it difficult to quantify its antagonist potency. However, it is worth noting that methysergide was a much weaker antagonist of 5-HT in the ear artery than in aorta, but was similar in potency against noradrenaline in these two preparations.

## Discussion

### *Monophasic and biphasic responses to agonists in the ear artery*

Noradrenaline produced transient monophasic responses in the ear artery, whereas 5-HT and methysergide produced prolonged biphasic responses. Because these prolonged responses resembled those obtained with tyramine and because 5-HT has been shown to act by releasing endogenous noradrenaline in some tissues (Innes, 1962), we sought evidence of such an action in the ear artery. Responses to both 5-HT and methysergide were unchanged in arteries from reserpine-treated rabbits, as found by Fozard (1973), indicating that neither drug was acting by releasing noradrenaline. The biphasic responses to 5-HT and methysergide probably occur because of the method of administration of the agonists (Apperley & Humphrey, 1976), the initial phase being caused by diffusion of drug through the intraluminal surface to the smooth muscle cells immediately after injection, and the second phase by diffusion through the extraluminal surface after the drug bolus has traversed the length of the artery and entered the fluid in which the artery is immersed. The response to noradrenaline is monophasic because noradrenaline is rapidly inactivated, mainly by Uptake$_1$, during diffusion through the extraluminal surface (de la Lande, Frewin & Waterson, 1967).

### *Receptors for noradrenaline and 5-hydroxytryptamine in aorta and ear artery*

The data obtained from this study provide new information on the distribution of (noradrenaline) $\alpha$-receptors and 5-HT-receptors in the rabbit aorta and ear artery. We used antagonists to classify the receptors involved and assumed that the pA$_2$ value corresponds to the affinity constant (Furchgott, 1972). This seems to be a reasonable assumption since, invariably, the noradrenaline and 5-HT dose-response curves were displaced to the right in a parallel manner by the antagonists, the Schild plots had slopes not significantly different from unity, and the possibility of non-specific antagonism had been eliminated.

The results show that noradrenaline and 5-HT act on different receptors in rabbit aorta. Phentolamine was a potent antagonist at the (noradrenaline) $\alpha$-receptors, and methysergide, pizotifen and cyproheptadine were potent antagonists at the 5-HT-receptors. The pA$_2$ values obtained with these antagonists agree well with those obtained in other tissues containing $\alpha$-receptors (Gulati, Parikh & Umar, 1968; Clineschmidt, Geller, Govier & Sjoerdsma, 1970; Patil, Fudge & Jacobwitz, 1972; Kennedy & Levy, 1974) and 5-HT-receptors (Gorlitz & Frey, 1973). Phentolamine was also a weak antagonist at 5-HT-receptors, and methysergide, pizotifen and cyproheptadine weak antagonists at $\alpha$-receptors.

The results in the ear artery were strikingly different from those in the aorta. The pA$_2$ values for each of the four antagonists in the ear artery were virtually identical against noradrenaline and 5-HT and this indicates that the agonists act on a common receptor to produce vasoconstriction. Furthermore, since the pA$_2$ values for the four antagonists in the ear artery were close to those obtained on $\alpha$-receptors in the aorta, we conclude that the common receptor is an $\alpha$-receptor and that the ear artery does not contain specific 5-HT-receptors. These conclusions are consistent with the finding that noradrenaline and 5-HT had similar potencies at their specific receptors in the aorta but that 5-HT was about 700 times less potent than noradrenaline in the ear artery.

### *Analysis of the actions of methysergide*

This insight into the distribution of $\alpha$-receptors and 5-HT-receptors in rabbit aorta and ear artery helps in the interpretation of the various actions of methysergide. As described above, methysergide was a potent antagonist at 5-HT-receptors and a weaker antagonist at $\alpha$-receptors in rabbit aorta. Methysergide also possesses very weak agonistic action in this preparation and this appears to be mediated through $\alpha$-receptors since it was antagonized by phentolamine at concentrations which did not affect 5-HT.

The finding that the constrictor response to methysergide was even weaker than normal in strips previously incubated with noradrenaline, but was unchanged in strips previously incubated with 5-HT, is consistent with an action at $\alpha$-receptors. It is possible that the prior exposure to noradrenaline causes desensitization of $\alpha$-receptors which reduces the response to methysergide, a weak partial agonist. Thus, methysergide acts as a potent antagonist at 5-HT-receptors and as a weak partial agonist at $\alpha$-receptors in rabbit aorta.

Methysergide was also a partial agonist compared to noradrenaline in the ear artery. Its vasoconstrictor action was antagonized by phentolamine, pizotifen and cyproheptadine, with pA$_2$ values virtually identical to those obtained against noradrenaline and 5-HT. This shows that methysergide activates $\alpha$-

receptors in this tissue. Fozard (1973) originally reported that the action of methysergide was unaffected by phentolamine, in direct contrast to our results, but this major discrepancy has now been resolved since Fozard (1976) recently reported that phentolamine does antagonize methysergide. Methysergide antagonized the actions of the more potent $\alpha$-receptor agonists noradrenaline and 5-HT, as would be expected with a partial agonist. Thus, methysergide acts as a partial agonist at $\alpha$-receptors in the ear artery; it is only a weak antagonist of 5-HT because this preparation does not contain specific 5-HT-receptors.

Methysergide was a better agonist in the ear artery than in the aorta. A possible explanation is that the relationship between stimulus and response is different in the two tissues. The potency of a partial agonist would be particularly susceptible to a difference of this sort (Furchgott, 1972; Jenkinson, 1973). Hence, if the stimulus required for the same fractional response was higher in the aorta than in the ear artery then the activity of a partial agonist such as methysergide would be less pronounced in the former preparation.

Our results confirm previous reports (de la Lande, Cannell & Waterson, 1966; Fozard, 1973; Carrol, Ebeling & Glover, 1974) that methysergide also sensitizes the rabbit ear artery to the vasoconstrictor action of noradrenaline. Fozard (1973) has suggested that sensitization results from sub-threshold vasoconstriction achieving threshold or greater levels in the presence of other vasoconstrictor agents. However, this explanation is unlikely to apply to the sensitization of the ear artery to noradrenaline by methysergide if, as our results show, these two agonists act at the same receptor. From classical drug receptor theory it can be predicted that a partial agonist such as methysergide would antagonize the effect of a full agonist such as noradrenaline (see Barlow, 1964; Ariens & Simonis, 1964). An alternative explanation is that methysergide blocks noradrenaline uptake; an amine-uptake blocking action has been reported in cat spleen (Owen, Herd, Kalberer, Pacha & Salzmann, 1971). On the other hand, there is no theoretical objection to the idea that potentiation of vasopressin responses by methysergide results from sub-threshold vasoconstriction, since these two agents act at different sites.

### *Similarity between $\alpha$-receptors and 5-hydroxytryptamine-receptors*

Our results demonstrate that 5-HT and 5-HT-receptor blocking drugs can act at $\alpha$-receptors and, conversely, that an $\alpha$-receptor blocking drug, phentolamine, can act at 5-HT-receptors. It was not possible in the two preparations examined to determine whether noradrenaline can act at 5-HT-receptors but Vane (1960) has shown that certain $\alpha$-receptor agonists contract rat stomach strip by an action at 5-HT-receptors. This cross reactivity is in accord with a large body of evidence (see Fozard, 1973 for references) and indicates that the two types of receptor, although distinct entities, have features in common. This can give rise to problems of interpretation unless detailed quantitative data are obtained. For example, Carroll *et al.* (1974), in a semi-quantitative study, showed that the vasoconstrictor action of methysergide in rabbit ear artery was abolished by pizotifen and concluded that methysergide acts at 5-HT-receptors. Our analysis shows that this interaction actually takes place at $\alpha$-receptors. Indeed, it is ironic that the rabbit ear artery, which is frequently used for the study of 5-HT agonist-antagonist interactions, does not contain specific 5-HT-receptors.

### *Relevance to migraine therapy*

Since three of the drugs examined in this study, namely methysergide, pizotifen and cyproheptadine, are used in the treatment of migraine, it is appropriate to consider whether our results have any bearing on their mode of action. Pizotifen and cyproheptadine were more potent than methysergide as 5-HT-receptor blocking drugs in rabbit aorta yet are less effective in the treatment of migraine (Lance, Anthony & Somerville, 1970). These findings are in accord with a growing concensus of opinion that antagonism of the vascular actions of 5-HT may not be relevant to the effectiveness of anti-migraine drugs. Fozard (1975) suggested that it might well be the sedative properties of cyproheptadine and the combined sedative and anti-depressant properties of pizotifen, rather than their anti-5-HT properties, that benefit the migraine sufferer because they alleviate the stress symptoms which may trigger an attack (Lance, 1973). The ability to mimic the vasoconstrictor action of 5-HT is now considered to be an important property of specific anti-migraine drugs (Saxena, 1972). Methysergide effectively mimics the vasoconstrictor action of 5-HT in the rabbit ear artery and initially we thought that this might be the basis of its therapeutic effect. However, we subsequently rejected this hypothesis because this action of methysergide, in the ear artery at least, occurs at higher concentrations than could be achieved after administration of therapeutic doses of the drug (estimated maximum blood concentration of about $10^{-7}$ mol/litre). Thus, it does not seem that the $\alpha$-adrenoceptor stimulation produced by methysergide in the ear artery can explain its therapeutic action in migraine. However, it may be that the ear artery is unrepresentative of the external carotid vasculature in general or that the rabbit differs from other species. For example, we have recently shown that very low concentrations of methysergide (threshold $10^{-8}$ mol/l) constrict the dog isolated ear artery (Apperley, Humphrey & Levy, unpublished observations), and Saxena (1974) has shown that low doses of methysergide selectively increase the resistance of the dog carotid vascular bed *in vivo*. We are currently investigating the mechanisms involved in these actions of methysergide.

We are very grateful to Miss Marion Philp and Miss Janet Healey for diligent and skilled technical assistance. Sandoz, Basle, kindly supplied samples of methysergide and pizotifen and Merck, Sharp and Dohme, a sample of cyproheptadine.

**References**

APPERLEY, EIRA & HUMPHREY, P.P.A. (1976). A proposed mechanism for the biphasic vasoconstrictor response to serotonin and methysergide in the rabbit ear artery. *J. Pharm. Pharmac.* In press.

APPERLEY, EIRA, HUMPHREY, P.P.A. & LEVY, G.P. (1974). Receptors for 5-hydroxytryptamine and noradrenaline in rabbit aorta and central ear artery. *Br. J. Pharmac.*, **52**, 131–132P.

ARIENS, E.J. & SIMONIS, A.N. (1964). Drug receptor interaction: interaction of one or more drugs with one receptor system. In *Molecular Pharmacology*, vol. 1, pp. 119–286, ed. Ariens, E.J., New York: Academic Press.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48–58.

BARLOW, R.B. (1964). *Introduction to Chemical Pharmacology*, pp. 1–26, 2nd edition. London: Methuen.

CARROLL, P.R., EBELING, P.W. & GLOVER, W.E. (1974). The responses of the human temporal and rabbit ear artery to 5-hydroxytryptamine and some of its antagonists. *Aust. J. exp. Biol. med. Sci.*, **52**, 813–823.

CLINESCHMIDT, B.V., GELLER, R.G., GOVIER, W.C. & SJOERDSMA, A. (1970). Reactivity to norepinephrine and nature of the alpha adrenergic receptor in vascular smooth muscle of a genetically hypertensive rat. *Eur. J. Pharmac.*, **10**, 45–50.

FARMER, J.B. (1966). The biphasic response of an isolated artery to tyramine. *Br. J. Pharmac. Chemother.*, **28**, 340–347.

FOZARD, J.R. (1973). Drug interactions on an isolated artery. In *Background to Migraine*, **5**, pp. 150–169. ed. Cumings, J.N., London: Heinemann.

FOZARD, J.R. (1975). The animal pharmacology of drugs used in the treatment of migraine. *J. Pharm. Pharmac.*, **27**, 297–321.

FOZARD, J.R. (1976). Comparative effects of four migraine prophylactic drugs on an isolated extracranial artery. *Eur. J. Pharmac.*, **36**, 127–139.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Catecholamines, Handb. exp. Pharmac.* N.S. Vol. 33, pp. 288–289. ed. Blaschko, H. & Muscholl, E. Berlin and Heidelberg: Springer-Verlag.

FURCHGOTT, R.F. & BHADRAKOM, S. (1953). Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. *J. Pharmac. exp. Ther.*, **108**, 129–143.

GORLITZ, B.D. & FREY, H.H. (1973). Comparison of the blocking effects of antagonists of adrenaline and 5-hydroxytryptamine on their mutual receptors. *J. Pharm. Pharmac.*, **25**, 651–653.

GULATI, O.D., PARIKH, H.M. & UMAR, M.S. (1968). Receptors for noradrenaline and histamine in the rabbit's posterior vena cava. *Br. J. Pharmac. Chemother.*, **32**, 87–95.

INNES, I.R. (1962). An action of 5-hydroxytryptamine on adrenaline receptors. *Br. J. Pharmac. Chemother.*, **19**, 427–441.

JENKINSON, D.H. (1973). Classification and properties of peripheral adrenergic receptors. *Br. med. Bull.*, **29**, 142–147.

KENNEDY, I. & LEVY, G.P. (1974). Combined $\alpha$ and $\beta$-adrenoceptor blocking drug, AH 5158: further studies on specificity and $\alpha$-adrenoceptor blocking action. *Proc. Aust. physiol. pharmac. Soc.*, **5**, No. 2, 216–217.

LANCE, J.W. (1973). The pathogenesis of migraine. In *Mechanism and Management of Headache*, pp. 113–133, 2nd edition. London: Butterworths.

LANCE, J.W., ANTHONY, M. & HINTERBERGER, H. (1970). Migraine and 5-hydroxytryptamine. In *Background to Migraine*, pp. 155–161. London: William Heinemann Medical Books.

LANCE, J.W., ANTHONY, M. & SOMERVILLE, B. (1970). Comparative trial of serotonin antagonists in the management of migraine. *Br. med. J.*, **2**, 327–330.

DE LA LANDE, I.S., CANNELL, V.A. & WATERSON, J.G. (1966). The interaction of serotonin and noradrenaline on the perfused artery. *Br. J. Pharmac. Chemother.*, **28**, 255–272.

DE LA LANDE, I.S., FREWIN, D. & WATERSON, J.G. (1967). The influence of sympathetic innervation on vascular sensitivity to noradrenaline. *Br. J. Pharmac. Chemother.*, **31**, 82–93.

DE LA LANDE, I.S. & RAND, M.J. (1965). A simple isolated nerve-blood vessel preparation. *Aust. J. exp. Biol. med. Sci.*, **43**, 639–656.

OWEN, D.A.A., HERD, J.K., KALBERER, F., PACHA, W. & SALZMANN, R. (1971). The influence of ergotamine and methysergide on the storage of biogenic amines. *Proc. Int. Headache Symposium*, Elsinore, Denmark. pp. 153–161.

PATIL, P.N., FUDGE, K. & JACOBWITZ, D. (1972). Steric aspects of adrenergic drugs. XVIII $\alpha$-adrenergic receptors of mammalian aorta. *Eur. J. Pharmac.*, **19**, 79–87.

SAXENA, P.R. (1972). The effects of antimigraine drugs on the vascular responses by 5-hydroxytryptamine and related biogenic substances on the external carotid bed of dogs: possible pharmacological implications to their antimigraine action. *Headache*, **12**, 44–54.

SAXENA, P.R. (1974). Selective vasoconstriction in carotid vascular bed by methysergide: possible relevance to its antimigraine effect. *Eur. J. Pharmac.*, **27**, 99–105.

VANE, J.R. (1960). The actions of sympathomimetic amines on tryptamine receptors. In *Adrenergic Mechanisms*, pp. 356–372. ed. Vane, J.R., Wolstenholme, A.E.W. & O'Connor, M. London: Churchill.

*(Received January 22, 1976.*
*Revised April 30, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 223–228

# EFFECT OF INDOMETHACIN AND PROSTAGLANDIN ON THE SMOOTH MUSCLE CONTRACTING ACTIVITY OF ANGIOTENSIN AND OTHER AGONISTS

JEANNINE ABOULAFIA, GUN B. MENDES, MARIA E. MIYAMOTO,
A.C.M. PAIVA & THEREZINHA B. PAIVA
Department of Biophysics and Physiology, Escola Paulista de Medicina,
04023 São Paulo, S.P., Brazil

1 Indomethacin had an equal inhibitory effect on the response of the guinea-pig isolated ileum to angiotensin II (angiotensin), bradykinin, histamine and acetylcholine. This effect did not seem to result from inhibition of prostaglandin synthesis, as it did not depend on the time of treatment with indomethacin.

2 Prostaglandin $E_2$ (prostaglandin) potentiated the responses of the guinea-pig ileum to angiotensin, bradykinin, histamine and acetylcholine without significant differences in the effects observed.

3 In the rabbit isolated mesenteric and coeliac arteries, indomethacin had an equal potentiating effect on the responses to angiotensin and to adrenaline. In these organs pre-incubation with indomethacin was necessary for the effect to be observed, and this effect lasted for 2 h or more after that drug was removed from the medium.

4 No cross-tachyphylaxis between angiotensin and adrenaline was observed in the rabbit mesenteric and coeliac arteries.

5 It is concluded that the effects of indomethacin and prostaglandin on the response of the guinea-pig ileum to the four agonists result from an action on the smooth muscle contractile mechanism *per se* rather than from an inhibitory action on the release of endogenous prostaglandin produced by the four agonists.

6 The results with the rabbit isolated arteries indicate that tachyphylaxis to angiotensin in these organs is not caused by prostaglandin release.

## Introduction

It has been postulated that prostaglandin release participates in the smooth muscle contraction induced by a number of agonists. Vane & Williams (1972) suggested that intramural generation of a prostaglandin contributes to the contractions induced by oxytocin in the rat isolated uterus.

Indomethacin, an inhibitor of prostaglandin biosynthesis (Vane, 1971), depresses responses of the rat uterus to bradykinin (Barabé, Park & Regoli, 1975) and angiotensin II (Baudouin-Legros, Meyer & Worcel, 1974). This has been interpreted as evidence that prostaglandin release is involved in the contraction of uterine smooth muscle by these peptides, although under oestrogen domination the uterus is more sensitive to these agonists, but less sensitive to prostaglandins (Weeks, 1972).

In vascular smooth muscles, in which prostaglandins usually produce relaxation (Weeks, 1972), prostaglandin release has been implicated in tachyphylaxis to angiotensin because this phenomenon was partly supressed by indomethacin (Aiken, 1974). However, in the atropinized guinea-pig isolated ileum neither the onset of tachyphylaxis to angiotensin nor recovery from it were affected by indomethacin (Paiva, Mendes & Paiva, 1976). On the other hand, Chong & Downing (1973, 1974) described a selective inhibition by indomethacin of angiotensin-induced contractions of the guinea-pig ileum, which was reversed by prostaglandin. They interpreted this as evidence that prostaglandin mediates angiotensin's action. We have not been able to confirm the selective nature of this inhibition, but have obtained evidence for a non-selective action of indomethacin in both guinea-pig ileum and rabbit arteries.

## Methods

Guinea-pigs of either sex weighing 150–200 g were deprived of food for 24 h and killed by decapitation. A 15 cm segment of terminal ileum was removed, and washed with Tyrode solution at room temperature. A

4 cm portion was then suspended in a 5 ml chamber containing Tyrode solution at 37°C, bubbled with air. Under these conditions the pH of the medium was 8.0. Isotonic contractions of longitudinal muscle were recorded on a smoked drum by a frontal writing lever under 1 g load and six-fold magnification.

Male albino rabbits of approximately 5 kg body weight were stunned and killed by exsanguination, the coeliac and mesenteric arteries were removed immediately, and cut into helical strips 2–3 mm wide and approximately 2 (coeliac) or 4 (mesenteric) cm long. The strips were suspended in Tyrode solution at 37°C under the same conditions used for the guinea-pig ileum, except that 95% $O_2$ and 5% $CO_2$ were bubbled through the solution, resulting in a pH of 7.4.

### *Solution and drugs*

The composition of the Tyrode solution was (mM): NaCl 140, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.53, $NaHCO_3$ 12, $NaH_2PO_4$ 0.36 and glucose 5.6.

[5-Isoleucine]-angiotensin II (angiotensin) (Paiva, Goissis, Juliano, Miyamoto & Paiva, 1974) and bradykinin (Tominaga, Stewart, Paiva & Paiva, 1975) were synthetic products made in this laboratory. Other drugs used were: histamine dihydrochloride (California Corporation for Biochemical Research), acetylcholine chloride (Sigma), adrenaline bitartrate (Fluka), indomethacin (Merck, Sharp & Dohme) and prostaglandin $E_2$ (Upjohn). Indomethacin solutions were prepared just before use by dissolving the drug in a small volume of saturated aqueous sodium carbonate, diluting to the desired volume with Tyrode solution, and adjusting to pH 8.0. Concentrated prostaglandin solutions (1 mg/ml) in 25% ethanol were stored frozen and diluted with Tyrode solution immediately before use. The vehicles used for the administration of indomethacin and prostaglandin had no detectable effect on the response to the isolated organs to agonists.

## Results

### *Guinea-pig ileum*

The effect of indomethacin or prostaglandin on the response to angiotensin, bradykinin, histamine and acetylcholine, was studied by determining dose-response curves to the agonists before and after treatment with the two drugs. Independent experiments were performed for each agonist, using preparations responding constantly to a submaximally effective concentration administered at 5 min intervals. The responses to 4 concentrations of agonist (producing 20%–80% maximum contraction) were then recorded. After treatment with 56 μM indomethacin for 45 min, and in the continuing presence of that drug, new dose-response curves were obtained. The ratio of the concentrations that produced 50% of maximum response after and before treatment with indomethacin was determined by interpolation (Table 1).

To study the effect of indomethacin on the maximum contractions produced by each agonist, independent experiments were conducted with fresh tissues responding constantly to small, submaximally effective, doses of agonist administered at 5 min intervals and with a 90 s contact time. The organ was treated once with a dose of the agonist 15 times higher than that producing 50% of maximum response. This supramaximal dose was 0.09 μM for bradykinin, 1 μM for histamine, 0.03 μM for angiotensin and 1.2 μM for acetylcholine. It was followed by incubation with 56 μM indomethacin for 45 min, and another administration of the same supramaximal dose. This procedure was adopted to avoid tachyphylaxis to high doses of angiotensin. The reduction in the response to the second supramaximal dose was expressed as a percentage of the pre-indomethacin maximum response (Table 1). In similar experiments, done with 8.4 μM indomethacin, no significant

**Table 1** Effect of indomethacin (56 μM) on the isotonic response of the guinea-pig isolated ileum to four agonists

| *Agonist* | *Dose-ratio** | *% Inhibition of maximum response* |
|---|---|---|
| Angiotensin | 2.03 ± 0.34 (7) | 17.8 ± 3.6 (8) |
| Bradykinin | 2.35 ± 0.39 (6) | 13.4 ± 2.8 (6) |
| Histamine | 3.31 ± 1.59 (6) | 12.3 ± 2.7 (7) |
| Acetylcholine | 2.45 ± 0.58 (7) | 16.5 ± 4.4 (8) |

* Ratio of the concentrations producing 50% of maximum response after and before treatment with indomethacin. Mean ± s.d. (*n*).

**Table 2** Effect of prostaglandin (28 nM) on the isotonic response of the guinea-pig isolated ileum to four agonists

| *Agonist* | *Dose-ratio** | *% Potentiation of maximum response* |
|---|---|---|
| Angiotensin | 0.54 ± 0.09 (5) | 6.2 ± 2.5 (4) |
| Bradykinin | 0.31 ± 0.06 (5) | 9.9 ± 2.3 (4) |
| Histamine | 0.44 ± 0.08 (4) | 5.4 ± 1.3 (4) |
| Acetylcholine | 0.37 ± 0.09 (4) | 10.3 ± 3.2 (4) |

* Ratio of the concentrations producing 50% of maximum response after and before treatment with prostaglandin. Mean ± s.d. (*n*).

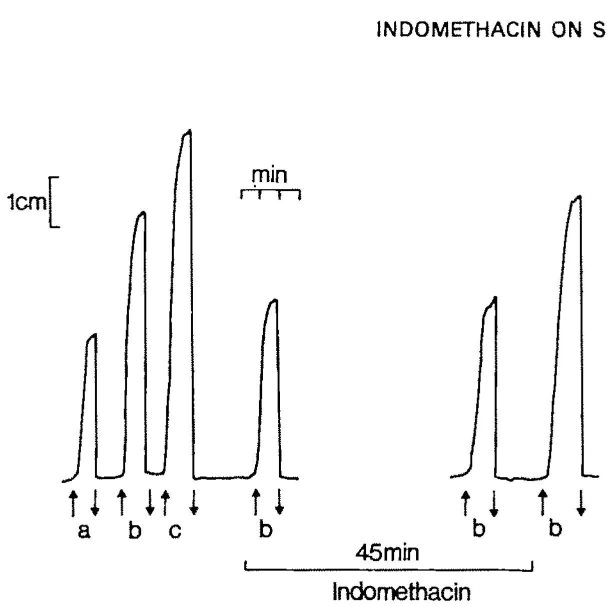


**Figure 1** Responses of the guinea-pig isolated ileum to angiotensin: a, 0.4 nM; b, 0.8 nM; c, 1.6 nM. Upward arrows indicate additions and downward arrows indicate washout and interruption of kymograph movement for 3 minutes. Normal Tyrode solution was replaced by one containing 56 µM indomethacin 30 s before addition of the fourth dose and restored 30 s before last dose.

inhibition of any of the four agonists was detected.

Each experiment to obtain the dose-ratio or percentage inhibition of maximum response was paired with a control without indomethacin. These showed no significant changes.

The effect of prostaglandin $E_2$ on the response of the guinea-pig ileum was studied using the same protocol (Table 2).

The inhibition of the response to the four agonists studied did not depend on the time of incubation with indomethacin, in the range 30 s to 45 minutes. The normal response of the isolated organ to these agonists was rapidly recovered after removal of indomethacin. This was reproducibly observed in at least three experiments with each agonist and is illustrated in Figure 1, for angiotensin.

*Rabbit mesenteric and coeliac arteries*

Angiotensin administered repeatedly, at 45 min intervals, to either the mesenteric or the coeliac artery, caused tachyphylaxis (Figure 2). This did not occur with adrenaline. However, treatment with 8.4 µM indomethacin for 30 min potentiated the responses of both agonists in both arteries. Figures 3 and 4 are representative of at least 6 experiments in each case. The potentiation was long-lasting, and was found to be present in some degree for up to 2 h after removal of indomethacin. Furthermore, in contrast to the guinea-pig ileum, previous incubation with indomethacin for 30 min was necessary for inhibition to occur.

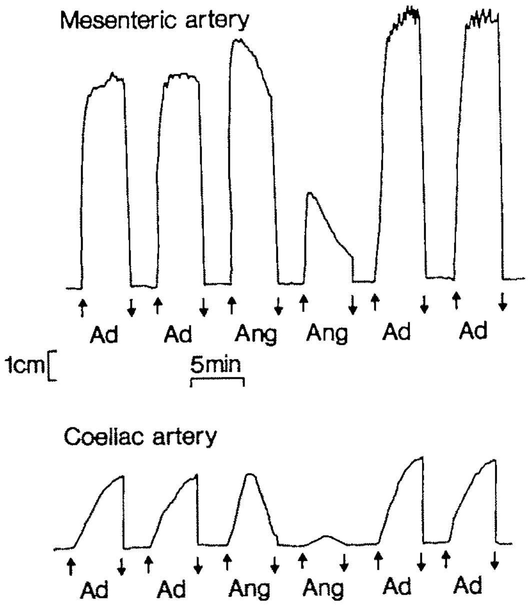


**Figure 2** Responses of the rabbit's mesenteric and coeliac arteries to repeated administration of adrenaline (Ad) and angiotensin (Ang). Upward arrows indicate additions and downward arrows indicate washout and interruption of kymograph movement for 3 minutes. Intervals between additions were 10 minutes.

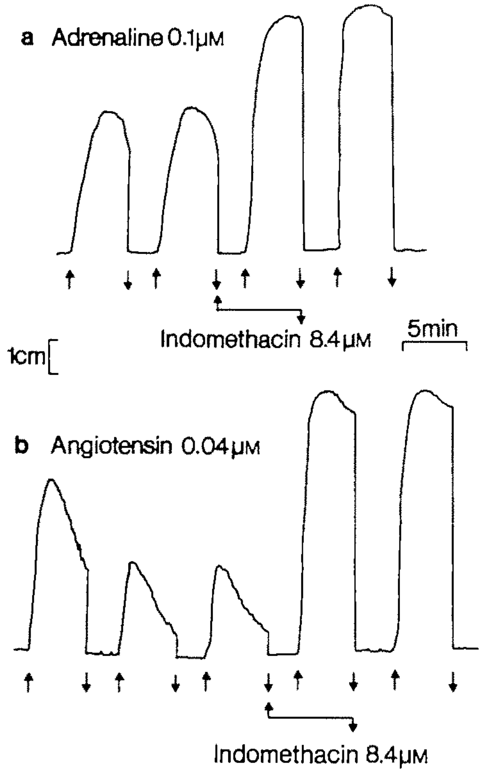


**Figure 3** Responses of the rabbit isolated mesenteric artery to sub-maximal doses of adrenaline and angiotensin administered at 45 min intervals. Upward arrows indicate additions and downward arrows indicate washout and interruption of kymograph movement for 38 minutes. Normal Tyrode solution was replaced by one containing 8.4 μM indomethacin 30 min before third (a) or fourth (b) dose, and restored 45 min before last dose.

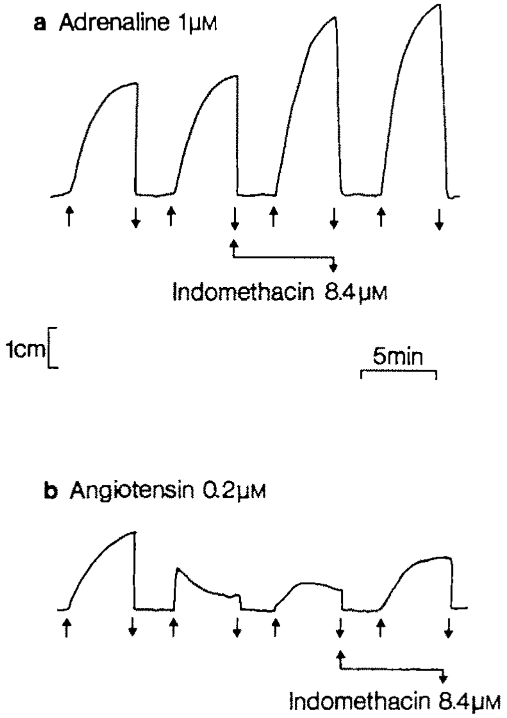


**Figure 4** Responses of the rabbit isolated coeliac artery to sub-maximal doses of adrenaline and angiotensin. Experimental details as in Figure 2.

## Discussion

### *Guinea-pig ileum*

Treatment of the guinea-pig isolated ileum with indomethacin inhibited contractions to angiotensin, but this effect was less intense than that described by Chong & Downing (1973) and was not selective for angiotensin. The dose-ratios and the inhibition of maximum response (Table 1) were not significantly different for the four agonists that were studied. Similarly, the potentiation by prostaglandin was non-selective, as shown in Table 1.

These results may indicate either that prostaglandin release mediates the action of the four agonists studied, probably at a common step in the excitation-contraction chain of events, or that prostaglandin and indomethacin have opposite effects on the contractile properties of the guinea-pig ileum, which affect the response to all agonists.

We believe that the experiment illustrated in Figure 1 favours the second hypothesis, since the effect of indomethacin occurred too quickly (30 s) to result from inhibition of prostaglandin synthesis. In particular the fast (30 s) return to normal response after the removal of indomethacin argues against this, since indomethacin inhibition appears to be at an early step of the synthetic pathway (Smith & Lands, 1971). It must also be pointed out that the concentrations of indomethacin needed to depress the response to the agonists were higher than those thought necessary to inhibit prostaglandin biosynthesis. Our finding that 8.4 μM indomethacin did not cause significant angiotensin inhibition is not in agreement with the report, by Kadlec, Masek & Seferna (1974), that 1 μM indomethacin depressed the response of the guinea-pig ileum to that agonist. However these authors do not state whether adequate care was taken to avoid tachyphylaxis to angiotensin, which might be enhanced by the lower pH of the Krebs solution that they used instead of the usual Tyrode (Paiva, Juliano, Nouailhetas & Paiva, 1974). It should be noted that they also report more than 100-fold loss of sensitivity to angiotensin in the presence of 0.4 μM atropine, while, under the usual conditions, 1 μM atropine only reduces the effect of angiotensin by 40–70% (Khairallah & Page, 1961; Robertson & Rubin, 1962).

Further evidence against prostaglandin mediation is the finding that guinea-pig ileum, in which response to prostaglandins was reduced by a dibenzoxazepine hydrazide derivative (SC-19220), responded normally to bradykinin and acetylcholine (Sanner, 1969) as well as to histamine (Ambache, Verney & Zar, 1970).

The available evidence thus indicates that the inhibitory action of indomethacin is probably due to an effect on one of the steps of the excitation-contraction coupling that is common to different stimuli. A similar conclusion was reached by Northover (1971), who worked with the guinea-pig isolated stomach; he observed that as widely different stimuli as electrical stimulation and histamine were inhibited by very large concentrations of indomethacin. This author also presented evidence linking indomethacin inhibition to diminished calcium uptake by the target cells.

The fact that prostaglandin potentiated the responses to the different agonists (Table 2) also points to an effect at a common link in the excitation-contraction chain of events. This might involve a calcium displacement mechanism, as postulated for similar effects in other smooth muscles (Clegg, Hall & Pickles, 1966; Strong & Bohr, 1967; Eagling, Lovell & Pickles, 1972; Villani, Chiarra, Cristalli & Piccinini, 1974) and for prostaglandin actions in other tissues (Ramwell & Shaw, 1970).

### *Rabbit arteries*

The experiments illustrated in Figures 3 and 4, carried out under the same conditions as those described by Aiken (1974), confirm his finding that indomethacin reverses angiotensin tachyphylaxis. However, this effect appears to be due to a non-selective potentiation since adrenaline, which shows no tachyphylaxis, was also potentiated. The possibility must be considered that prostaglandin release may occur with both agonists and that potentiation by indomethacin may be the result of inhibition of prostaglandin synthesis. This hypothesis is based on the non-specific relaxation of the muscle by prostaglandin (Aiken, 1974), and depends on the possibility of the presence of released prostaglandins for relatively long periods in the organ's biophase. If this were the case, however, one might expect that an organ rendered tachyphylactic to angiotensin would not respond fully to adrenaline. This was not observed in the experiments illustrated in Figure 2, where it is also shown that the isolated arteries respond normally to angiotensin after being challenged with adrenaline. Furthermore, we have confirmed Aiken's (1974) observation that tachyphylaxis to angiotensin in the rabbit mesenteric and coeliac arteries occurs even in the presence of indomethacin, although not as severely as in its absence.

Our results indicate that, in the systems that we have examined, tachyphylaxis to angiotensin is not caused by prostaglandin release. We believe that this is also supported by Aiken's (1974) observation that tachyphylaxis produced by repeated administration of angiotensin at shorter intervals (5–10 min) was not affected by inhibitors of prostaglandin synthesis. He interpreted this finding as indicative of a dual mechanism for tachyphylaxis, a short-lasting and a long-lasting one, of which only the latter would be dependent on prostaglandin release. However, it seems unlikely that the prostaglandin released by angiotensin would inhibit the response of a subsequent dose administered 45 min later but not one administered 10 min later. We conclude therefore that prostaglandin does not participate in the mechanism of angiotensin tachyphylaxis in rabbit arteries. We have reached a similar conclusion for the guinea-pig ileum (Paiva *et al.*, 1976).

The need for an incubation period with indomethacin and the persistence of its effect after washout suggests that, in the rabbit mesenteric and coeliac arteries, this drug might act through inhibition of prostaglandin synthesis. A conclusion about the role of prostaglandin and the mechanism of indomethacin's effects on vascular smooth muscle contraction is difficult, because of the reported differences in behaviour towards different prostaglandins, complicated by species variations (Weeks, 1972), and by differences among large and small arteries from the same species (Strong & Bohr, 1967; Aiken, 1974). However, prostaglandins and indomethacin might affect the response of vascular smooth muscle through opposite effects on calcium translocation mechanisms, as proposed for the guinea-pig ileum. The available evidence indicates that calcium transport in vascular smooth muscles is directly affected by indomethacin (Northover, 1971) and prostaglandins (Greenberg, Kadowitz, Diecke & Long, 1973).

This work was done with the technical assistance of N.A. Mora and supported by grants from the Brazilian National Council for Scientific and Technological Development (CNPq), the São Paulo State Research Foundation (FAPESP) and the Financiadora de Estudos e Projetos (FINEP). The authors are grateful to Dr John E. Pike, of the Upjohn Co., Kalamazoo Mich, U.S.A. for supplying the prostaglandin $E_2$.

## References

AIKEN, J.W. (1974). Effects of prostaglandin synthesis inhibitors on angiotensin tachyphylaxis in the isolated coeliac and mesenteric arteries of the rabbit. *Pol. J. Pharmac. Pharm.*, **26**, 217–227.

AMBACHE, N., VERNEY, J. & ZAR, M.A. (1970). Evidence for the release of two atropine-resistant spasmogens from Auerbach's plexus. *J. Physiol., Lond.*, **207**, 761–782.

BARABÉ, J., PARK, W.K. & REGOLI, D. (1975). Application of drug receptor theories to the analysis of the myotropic effects of bradykinin. *Can. J. Physiol. Pharmac.*, **53**, 345–353.

BAUDOUIN-LEGROS, M.Y., MEYER, P. & WORCEL, M. (1974). Influence of prostaglandins and oestrous cycle on the spasmodic action of angiotensin II and oxytocin on rat uterus. *Br. J. Pharmac.*, **51**, 121–122P.

CHONG, E.K.S. & DOWNING, O.A. (1973). Selective inhibition of angiotensin-induced contractions of smooth muscle by indomethacin. *J. Pharm. Pharmac.*, **25**, 170–171.

CHONG, E.K.S. & DOWNING, O.A. (1974). Reversal by prostaglandin $E_2$ of the inhibitory effect of indomethacin on contractions of guinea-pig ileum induced by angiotensin. *J. Pharm. Pharmac.*, **26**, 729–730.

CLEGG, P., HALL, C. & PICKLES, V.R. (1966). The action of ketonic prostaglandins on the guinea-pig myometrium. *J. Physiol., Lond.*, **183**, 123–144.

EAGLING, E.M., LOVELL, H. & PICKLES, V.R. (1972). Interaction of prostaglandin $E_1$ and calcium in the guinea-pig myometrium. *Br. J. Pharmac.*, **44**, 510–516.

GREENBERG, S., KADOWITZ, P.J., DIECKE, F.P.J. & LONG, J.P. (1973). Effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on arterial and venous contractility and $^{45}$Ca uptake. *Archs. int. Pharmacodyn.*, **205**, 381–398.

KADLEC, O., MASEK, K. & SEFERNA, I. (1974). A modulatory role of prostaglandins in contractions of the guinea-pig ileum. *Br. J. Pharmac.*, **51**, 565–570.

KHAIRALLAH, P.A. & PAGE, I.H. (1961). Mechanism of action of angiotensin and bradykinin on smooth muscle *in situ*. *Am J. Physiol.*, **200**, 51–54.

NORTHOVER, B.J. (1971). Mechanism of the inhibitory action of indomethacin on smooth muscle. *Br. J. Pharmac.*, **41**, 540–551.

PAIVA, T.B., GOISSIS, G., JULIANO, L., MIYAMOTO, M.E. & PAIVA, A.C.M. (1974). Angiotensin-like and antagonistic activities of N-terminal modified [8-Leucine]angiotensin II peptides. *J. med. Chem.*, **17**, 238–241.

PAIVA, T.B., JULIANO, L., NOUAILHETAS, V.L.A. & PAIVA, A.C.M. (1974). The effect of pH on tachyphylaxis to angiotensin peptides in the isolated guinea pig ileum and rat uterus. *Eur. J. Pharmac.*, **25**, 191–196.

PAIVA, T.B., MENDES, G.B. & PAIVA, A.C.M. (1976). The specific desensitization (tachyphylaxis) of the guinea pig ileum to angiotensin II. *Am. J. Physiol.* (in press).

RAMWELL, P.W. & SHAW, J.E. (1970). Biological significance of the prostaglandins. *Recent Prog. Horm. Res.*, **26**, 139–187.

ROBERTSON, P.A. & RUBIN, D. (1962). Stimulation of intestinal nervous elements by angiotensin. *Br. J. Pharmac.*, **19**, 5–12.

SANNER, J.H. (1969). Antagonism of prostaglandin $E_2$ by 1-acetyl-2-(8-chloro-10,11-Dihydrodibenz [b,f][1,4]oxazepine-10-carbonyl)hydrazine (SC-19220). *Archs. int. Pharmacodyn.*, **180**, 46–56.

SMITH, W.L. & LANDS, W.E.M. (1971). Stimulation and blockade of prostaglandin biosynthesis. *J. biol. Chem.*, **246**, 6700–6704.

STRONG, C.G. & BOHR, D.F. (1967). Effects of prostaglandins $E_1$, $E_2$, $A_1$ and $F_{1\alpha}$ on isolated vascular smooth muscle. *Am. J. Physiol.*, **213**, 725–733.

TOMINAGA, M., STEWART, J.M., PAIVA, T.B. & PAIVA, A.C.M. (1975). Synthesis and properties of new bradykinin potentiating peptides. *J. med. Chem.*, **18**, 130–133.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. *Nature, New Biol.*, **231**, 232–235.

VANE, J.R. & WILLIAMS, K.I. (1972). Prostaglandin production contributes to the contractions of the isolated uterus. *Br. J. Pharmac.*, **45**, 146P.

VILLANI, F., CHIARRA, A., CRISTALLI, S. & PICCININI, F. (1974). Effect of $PGE_2$ on the turnover of calcium in rat uterus. *Experientia*, **30**, 532–534.

WEEKS, J.R. (1972). Prostaglandins. *A. Rev. Pharmac.*, **12**, 317–336.

*(Received February 9, 1976.*
*Revised April 20, 1976)*

*Br. J. Pharmac.* (1976), **58**, 229–237

# THE DENERVATED CREMASTER MUSCLE OF THE GUINEA-PIG AS A PHARMACOLOGICAL PREPARATION

M. MAUREEN DALE, AKGÜN EVINÇ[1] & PAULINE VINE
Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

**1** The pharmacological responses of both denervated and innervated cremaster muscle preparations from the guinea-pig have been investigated and compared. Acetylcholine (ACh) dose-response curves were obtained in both preparations. The affinity constants for (+)-tubocurarine and for atropine were calculated and indicated that the ACh receptors in both preparations were nicotinic.

**2** Histamine dose-response curves could be obtained only in the denervated preparation. The response was unaltered by metiamide, and the affinity constant for mepyramine fitted in with those previously obtained by others on ileum and trachea, indicating that the histamine receptors are $H_1$ in type.

**3** Sustained contractions were obtained to adrenaline and noradrenaline but not isoprenaline, with the denervated preparations.

**4** Schultz-Dale responses were obtained with denervated muscle in sensitized guinea-pigs. This preparation did not respond to 5-hydroxytryptamine, bradykinin, or the slow-reacting substance of anaphylaxis.

## Introduction

On denervation the pharmacological properties of skeletal muscle change, the receptor areas sensitive to acetylcholine (ACh) increase and, in the guinea-pig, sensitivity to other substances such as histamine and adrenaline may appear. Anaphylactic contractions, normally seen only in smooth muscle, have been described in guinea-pig denervated skeletal muscle and attributed to direct stimulation of the muscle by the antigen-antibody reaction (Alonso-de Florida, del Castillo, Gonzales & Sanchez, 1965). The exact mechanism of this anaphylactic contraction has not been clarified, and the receptors involved in the histamine responses have not previously been investigated.

In this preliminary study, the usefulness of an isolated cremaster muscle for studies on guinea-pig denervated skeletal muscle has been investigated and some of the responses obtained with denervated muscle have been compared with those of innervated preparations.

The effects of cholinomimetic drugs, anticholinesterases and ACh antagonists have been assessed and compared in denervated and innervated preparations, and the responses of denervated preparations to histamine, catecholamines and a number of other pharmacological agents have been investigated. Experiments on the nature of the histamine receptors have been carried out.

While this manuscript was in preparation, a paper appeared outlining some pharmacological properties of the cremaster muscle of the guinea-pig (Ninomiya, 1975). Ninomiya found that the guinea-pig cremaster manifested substantial contractions (2 g) with transmural stimulation and adrenaline 1 μg/ml produced an increase in tension of 300 mg. ACh and carbachol increased baseline tension by 200 mg and also increased spontaneous contractions in the tip of the muscle.

## Methods

Hartley guinea-pigs of 250 to 300 g were used for denervation and the cremaster muscle was used for experiments 2 to 6 weeks afterwards.

### *Denervation*

The abdomen was shaved and cleaned with alcohol, the guinea-pig anaesthetized with halothane and a midline incision made. The abdominal contents were expressed, wrapped in warm wet sterile gauze and pulled to one side to expose the posterior abdominal wall. The genito-femoral nerve was cut. In later operations a 5 mm portion was removed in order to be sure that regeneration would not occur. In some animals both cremaster muscles were denervated.

---

[1] Present address: c/o Institute of Pharmacology, Ege University, Faculty of Medicine, Bornova, Izmir, Turkey.

### *Dissection of the cremaster muscle preparation*

The skin was reflected from the abdominal wall and both testes. A midline incision was made through the abdominal muscles, extending caudally as far as the symphysis pubis. The testis was pushed back through the inguinal canal into the abdomen, the cremaster muscle being everted in the process. The spermatic cord was cut and the cremaster muscle was carefully dissected free by cutting through the abdominal wall at its base. Finally the testis was cut free from its attachment to the cremaster sac leaving a small tag of testicular tissue for the attachment of a ligature. At no stage was the cremaster muscle itself cut, but rather the structures adjacent to it, so as to minimize damage to the muscle.

### *Recording apparatus*

The muscle was set up in a 5 ml organ bath at 37°C in oxygenated Tyrode solution of the following composition (g/l): NaCl 8, KCl 0.2, $MgSO_4.7H_2O$ 0.26, $CaCl_2$ 0.396, $NaH_2PO_4.2H_2O$ 0.65, glucose 1.0 and $NaHCO_3$ 1.0. Isotonic contractions were recorded with a Servoscribe pen recorder, 0.5 g of tension being applied to the muscle. The recording apparatus was calibrated after each experiment by depressing the lever by measured amounts (2 mm, 5 mm, 10 mm) and recording the corresponding deflections of the pen recorder. Because there is a linear relationship between the excursion of the lever and the deflections on the pen recorder, the contractions of the muscle could also be expressed as a percentage of its length. With some agents, isometric contractions were recorded, with a Pye-Ether UFI transducer and Servoscribe recorder.

In general, contractile responses to drugs were expressed as a percentage of the maximum contraction produced by 0.1 M KCl.

### *Drugs*

The following drugs were used: acetylcholine chloride (Sigma Chemical Co.), adrenaline bitartrate (BDH), atropine sulphate (BDH), bradykinin (Sandoz), carbachol chloride (BDH), histamine dihydrogen phosphate (BDH), 5-hydroxytryptamine creatinine sulphate (Sigma Chemical Co.), isoprenaline sulphate (Burroughs Wellcome & Co.), mepyramine maleate (May & Baker), methacholine chloride (Sigma Chemical Co.), metiamide (Smith, Kline & French), neostigmine methanesulphonate (Koch-Light), noradrenaline hydrogen tartrate (levophed; Winthrop), ovalbumin (BDH), physostigmine sulphate (BDH), slow reacting substance of anaphylaxis (SRS-A)—an extract was obtained from Dr W.E. Brocklehurst (Biology Department, Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey), (+)-tubocurarine chloride (Sigma Chemical Co.).

The catecholamine solutions were made freshly just before use.

### *Calculation of affinity constants of competitive antagonists*

In some instances the affinity constant ($K_I$) of an antagonist was calculated from the dose-ratio (A/a) using the equation

$$A/a - 1 = K_I[I]$$

where A/a is the ratio of two concentrations of agonist which produce the same response with and without the antagonist, and [I] is the concentration of antagonist (Schild, 1949).

In some cases, where it was possible to obtain a series of parallel log dose-response curves with increasing concentrations of antagonist, the affinity constant was calculated from a Schild plot: a graph of log (A(a−1) against log [I] (Arunlakshana & Schild, 1959). In these cases it was possible to obtain a measure of the slope of the line, which equals 1 in a simple competitive antagonism.

## Results

### *Response to acetylcholine*

*Denervated preparations* The response to ACh was tested in seven denervated preparations. All responded with graded and readily reproducible contractions to concentrations of ACh in the range $2 \times 10^{-7}$ M to $10^{-5}$ M. An example is given in Figure 1a. This range is similar to that reported for the response of the denervated hemi-diaphragm of the rat (Elmqvist & Thesleff, 1960) and the gracilis muscle of the rat (Turkanis, 1969). The response and recovery after each dose was rapid, and a 2 or 3 min time cycle with a 20 s drug contact period gave satisfactory results with little loss of sensitivity except at high concentrations of the drug.

The maximum isotonic contractions produced by ACh were generally about 70% of the contraction produced by 0.1 M KCl. The maximum isometric contractions represented 2–5 g tension in different preparations which constituted 40–70% of the maximum tension obtained with 0.1 M KCl. The preparations were most sensitive between 2 and 4 weeks after denervation.

*Innervated preparations* Eight preparations were tested in the same way as the denervated preparations. All responded to ACh with graded and readily reproducible contractions to concentrations usually within the range $5 \times 10^{-6}$ to $5 \times 10^{-4}$ M (an example is given in Figure 1b). The muscles were about fifty times less sensitive than denervated preparations. The

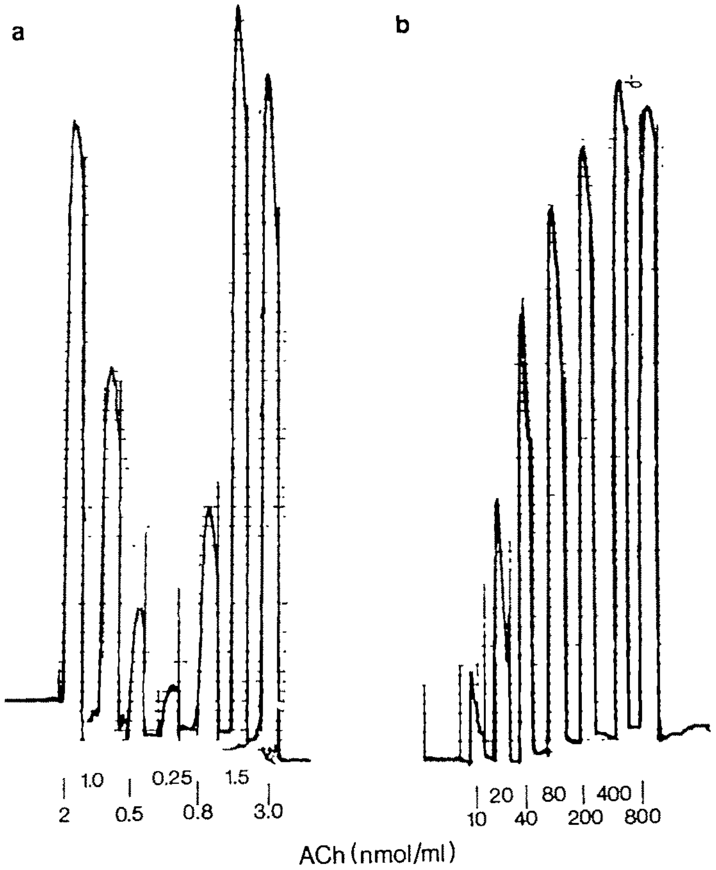


**Figure 1** Responses of the isolated cremaster muscle to acetylcholine (ACh). (a) The denervated preparation responded to concentrations from $2.5 \times 10^{-7}$ to $3 \times 10^{-6}$ M (0.25–3 nmol/ml), and (b) the innervated preparation to concentrations ranging from $10^{-5}$ to $8 \times 10^{-4}$ M (10–80 nmol/ml). The maximum ACh contraction was about 80% of the maximum contraction with 0.1 M KCl (which caused a 50% shortening of the muscle length).

magnitude of the contractions was similar to that obtained with denervated preparations although the innervated preparations gave a slightly less prolonged response than those that had been denervated. Desensitization was minimal except when very high doses of ACh were used, or when anticholinesterases were added to the bathing fluid.

### *The effect of anticholinesterases on acetylcholine responses*

*Denervated preparations* Langley & Kato observed in 1915 that anticholinesterases did not potentiate the effects of ACh on denervated frog gastrocnemius muscle. In the denervated cremaster muscle some potentiation was observed with physostigmine sulphate, $6 \times 10^{-7}$ M. A 6-fold potentiation was found in one experiment though in others the potentiation was only 3-fold. The fact that any potentiation at all was observed indicates that some cholinesterase is present.

*Innervated preparations* Physostigmine sulphate $6 \times 10^{-7}$ M and neostigmine methanesulphonate $6 \times 10^{-7}$ M both produced a 3- to 7-fold potentiation of the response to ACh. This was equivalent to the potentiation produced in the denervated preparations, but less consistent dose-response curves were obtained in the presence of the anticholinesterases, since marked desensitization occurred.

### *The effect of acetylcholine antagonists*

*Denervated preparations* Although most studies support the view that the new ACh receptors in denervated mammalian muscle are nicotinic (Elmqvist

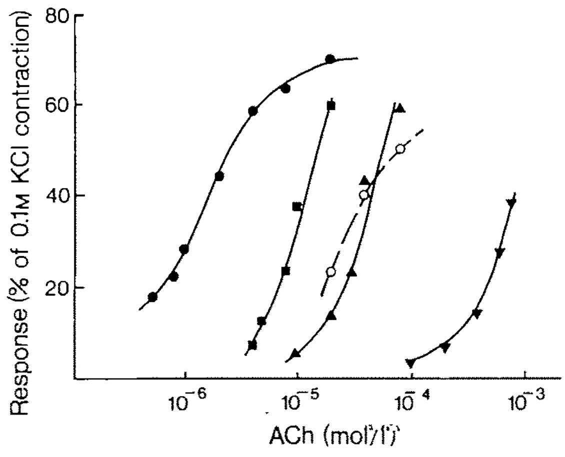


**Figure 2** Effect of (+)-tubocurarine (Tc) on the acetylcholine (ACh) response of denervated cremaster muscle: (●) ACh alone, (■) ACh+Tc $10^{-7}$ M, (▲) ACh+Tc $10^{-6}$ M, (▼) ACh+Tc $10^{-5}$ M, (○) partial recovery after washing out Tc.

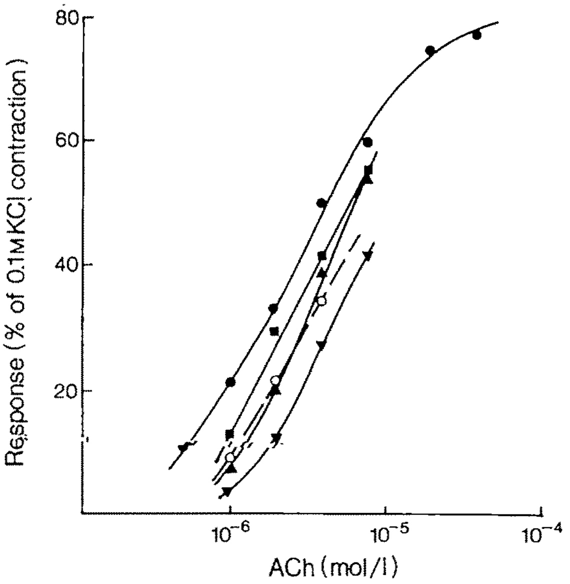


**Figure 3** Effect of atropine on the acetylcholine (ACh) response of denervated cremaster muscle: (●) ACh alone, (■) ACh+atropine $10^{-7}$ M, (▲) ACh+atropine $10^{-6}$ M, (▼) ACh+atropine $10^{-5}$ M, (○) recovery curve after washing out atropine.

& Thesleff, 1960; Beránek & Vyskočil, 1967), some evidence indicates that muscarinic receptors might also be present (Turkanis, 1969). The effects of a competitive antagonist for nicotinic receptors ((+)-tubocurarine) and a competitive antagonist for muscarinic receptors (atropine) were tested on the denervated cremaster muscle. When the log dose-response curves to ACh alone and in the presence of (+)-tubocurarine were plotted, there was a marked reversible parallel shift to the right with increasing doses of antagonist from $10^{-9}$ M to $10^{-5}$ M (see Figure 2). Atropine, on the other hand, had only a minimal effect even at $10^{-5}$ M, when tested concurrently on the contralateral denervated cremaster muscle of the same guinea-pig (see Figure 3).

There was some loss of viability of the preparations towards the end of the experiments as evidenced by the fact that recovery, after washing the antagonist out of the bath, was not complete. Using the values from Figure 2, a Schild plot was constructed, graphing log (A/a−1) against log [I]. The slope of the line which best fitted the points was 0.95 and the affinity constant for (+)-tubocurarine was $2 \times 10^7$ $M^{-1}$ ($pA_2 = 7.3$). This value is fairly similar to the figures reported for innervated guinea-pig skeletal muscle, which have been in the region of $9 \times 10^6$ $M^{-1}$ ($pA_2 = 7.0$) for diaphragm and $11 \times 10^6$ $M^{-1}$ ($pA_2 = 7.0$) for latissimus dorsi (Lu, 1970).

The affinity constant for atropine calculated from the dose-ratio given by the highest concentration of atropine was $2 \times 10^5$ $M^{-1}$ ($pA_2 = 5.3$) which compares with the $pA_2$ value of 4.2 for atropine on the frog rectus (see Arunlakshana & Schild, 1959).

These data indicate that in the denervated guinea-pig cremaster muscle, the ACh receptors are very similar to those in innervated guinea-pig skeletal muscle and are mainly if not entirely nicotinic.

*Innervated preparations* (+)-Tubocurarine produced reversible, dose-related shifts of the dose-response curve to ACh, whereas atropine had little or no effect. Examples are given in Figures 4 and 5. The affinity constant for tubocurarine obtained from a Schild plot was $8 \times 10^7$ $M^{-1}$ ($pA_2 = 7.9$), and the slope of the line was 1.12. This $pA_2$ value is rather higher than the figures reported by Lu (1970) for innervated guinea-pig skeletal muscle. The difference could again be due to the fact that our preparations lost sensitivity during the course of the experiment. This would be expected to give rather high dose-ratios.

### *The effect of other cholinomimetic agonists*

*Methacholine* The denervated muscle gave repeatable graded responses to methacholine in the concentration range $4 \times 10^{-4}$ to $4 \times 10^{-3}$ M. The responses were of similar size and time course to those elicited by ACh but methacholine was approximately 400

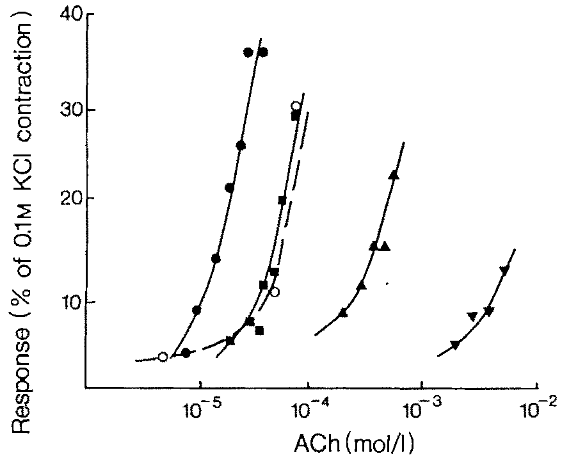


**Figure 4** Effect of (+)-tubocurarine (Tc) on the acetylcholine (ACh) response of innervated cremaster muscle: (●) ACh alone, (■) ACh+Tc $10^{-7}$ M, (▲) ACh+Tc $10^{-6}$ M, (▼) ACh+Tc $10^{-5}$ M, (O) partial recovery after washout of tubocurarine.

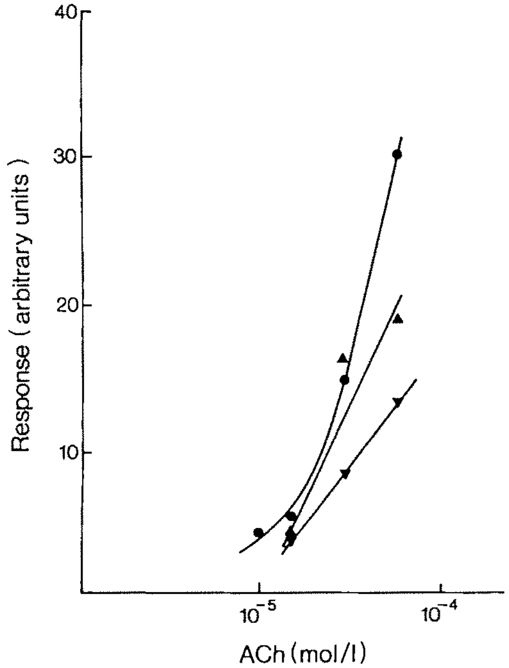


**Figure 5** The effect of atropine on the acetylcholine (ACh) response of innervated cremaster muscle: (●) ACh alone, (▲) ACh+atropine 4 × $10^{-7}$ M, (▼) ACh+atropine 2 × $10^{-6}$ M. Neostigmine 2 × $10^{-7}$ M was present in the bath fluid throughout the experiment. Responses in arbitrary units: 30 units is equivalent to approximately 60% of the contraction with 0.1 M KCl.

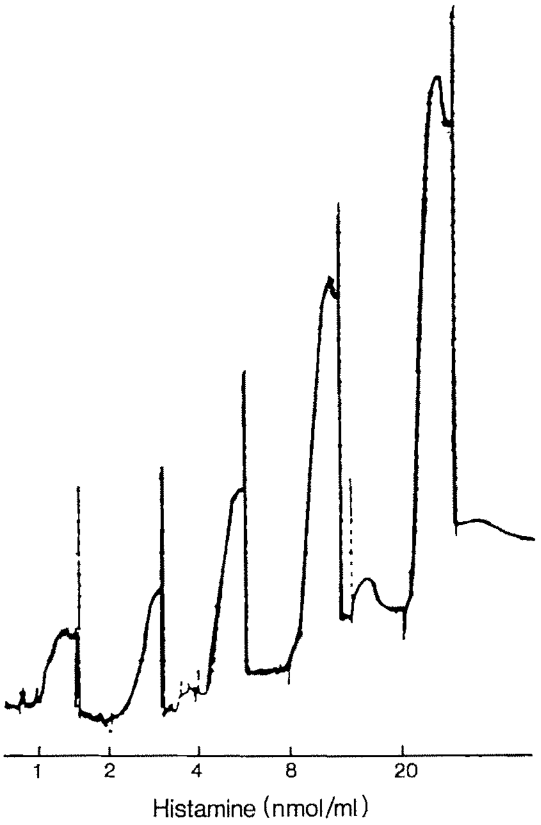


**Figure 6** Response of the denervated isolated cremaster muscle preparation to histamine. The maximum histamine contraction was about 40% of the maximum contraction obtained with 0.1 M KCl (which gave about 50% shortening of the muscle length).

times less potent. Methacholine has been reported to have an activity 180 times less than that of ACh on frog rectus (Barlow, 1964).

*Carbachol* Carbachol in the concentration range $10^{-6}$ to $10^{-5}$ M produced repeatable graded responses. It was roughly 3 times less potent than ACh on this preparation.

### *The response to histamine*

Contractions of the denervated cremaster muscle could be elicited with concentrations of histamine in the range $10^{-7}$ to $10^{-5}$ M. No responses were observed in innervated controls at concentrations up to $10^{-3}$ M. The response to histamine was slower than that to ACh, and smaller. The ACh contraction began

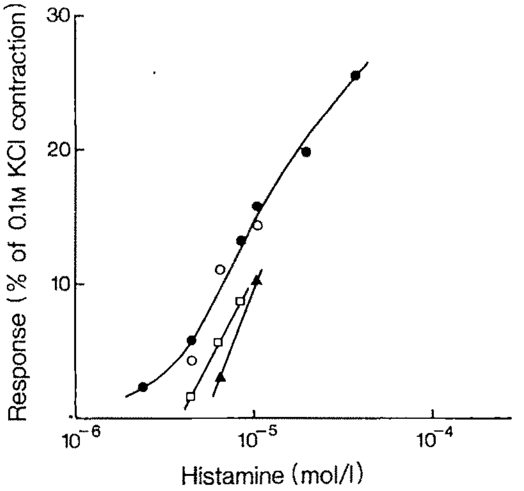


**Figure 7** The effect of metiamide on histamine-induced contractions of denervated cremaster muscle: (●) histamine alone, (○) histamine + metiamide $10^{-6}$ M, (□) histamine + metiamide $10^{-5}$ M, (▲) histamine + metiamide $10^{-4}$ M.

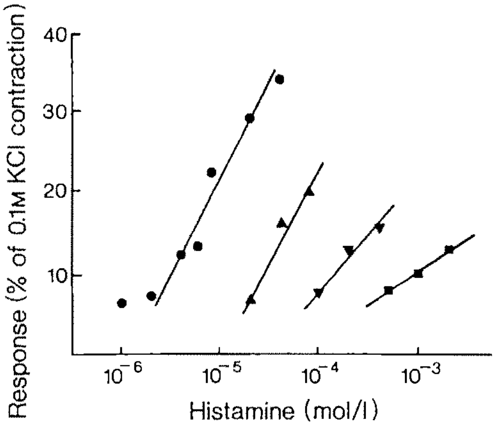


**Figure 8** Effect of mepyramine on histamine-induced contractions of denervated cremaster muscle: (●) histamine alone, (▲) histamine + mepyramine $2 \times 10^{-9}$ M, (▲) histamine + mepyramine $4 \times 10^{-9}$ M, (■) histamine + mepyramine $8 \times 10^{-9}$ M.

immediately after the drug was given and reached its peak in 20 s and on washing the drug out there was a rapid relaxation. With histamine there was a latency of 20–30 s before the response began, the contraction reached maximum 20–45 s later and recovery was very much slower. For experiments with histamine a drug contact time of 1 min was used, with a 15 min interval between doses. With higher concentrations full relaxation did not occur even after 15 minutes. Desensitization was a problem in most experiments with histamine. After the first fairly large contraction in response to a small dose, the sensitivity would drop and then remain steady. Then after about 10 responses it would decrease again unless a long rest period of about an hour was allowed. An example of the response to histamine is given in Figure 6.

The responses were small in magnitude compared with those to ACh. With isotonic recording the maximum histamine contractions were about 35% of the contracture produced by 0.1 M KCl.

Whereas the optimum time for eliciting responses to ACh was 2 to 4 weeks after denervation, the optimum time for histamine responses was 4 to 6 weeks after denervation. Earlier than this the muscle was relatively insensitive to histamine and later than this the muscle was usually too atrophied to give good responses.

### *The effect of histamine antagonists*

*Metiamide* This $H_2$-receptor antagonist appeared to have little or no effect on the response to histamine.

The dose-response curves for histamine alone and in the presence of metiamide $10^{-6}$ M were indistinguishable. The dose-response curve in the presence of $10^{-5}$ M and $10^{-4}$ M metiamide showed a slight shift to the right but this probably represented loss of sensitivity since no recovery occurred when the antagonist was washed out of the bath (see Figure 7).

*Mepyramine* This $H_1$-receptor antagonist produced a dose-related shift in the histamine dose-response curve. Mepyramine $10^{-9}$ M produced a parallel shift while higher doses produced non-parallel shifts (Figure 8). One possible interpretation is that mepyramine is a non-competitive antagonist in this system, but it is much more likely that the effect is due to non-specific loss of sensitivity of the tissue. Certainly there was poor recovery of the histamine response after washing the high doses of antagonist out of the bath.

If the dose-ratio given by the lowest concentration of mepyramine was used to calculate the affinity constant, the values obtained in 2 experiments were $6 \times 10^9$ $M^{-1}$ ($pA_2 = 9.7$) and $4 \times 10^9$ $M^{-1}$ ($pA_2 = 9.6$). These fall within the range reported for mepyramine in other tissues with $H_1$-receptors, which vary from $pA_2 = 9.1$ in the guinea-pig tracheal chain preparation (Arunlakshana & Schild, 1959) to $pA_2 = 9.8$ for guinea-pig ileum (Ash & Schild, 1966).

### *The response to catecholamines*

Adrenaline at a dose of $5 \times 10^{-6}$ M produced a slowly developing contracture over the course of 2 min or so.

It was not possible to obtain a dose-response curve because not only was the effect slow in wearing off, but in some cases the muscle continued to contract after the adrenaline was washed out of the bath. With isotonic recording, the peak response was usually about 30% of the 0.1 M KCl contracture (in one case it was 50%) and was sometimes little reduced after 20 minutes. Atropine and (+)-tubocurarine did not affect this contraction. Noradrenaline $5 \times 10^{-6}$ M also produced a slow contracture in two preparations in which it was tested. This reached 30% of the KCl maximum and also continued to increase after the drug was washed out.

Isoprenaline at $5 \times 10^{-6}$ M produced no effect and at $5 \times 10^{-5}$ M produced only a very tiny response, less than 5% of the KCl maximum.

### *Responses to other pharmacological agents*

Some substances thought to be involved in anaphylactic or other immunological responses which were tested on the denervated muscle but which had no effect were, 5-hydroxytryptamine (up to $10^{-3}$ M), bradykinin (up to 50 µg/ml) and slow-reacting substance of anaphylaxis.

### *Response of sensitized muscle to antigen*

The denervated cremaster muscle manifested Schultz-Dale responses. In five preparations from guinea-pigs sensitized to ovalbumin, ovalbumin 100 µg/ml elicited a contraction about 25% of the 0.1 M KCl maximum. The contraction had a long latency and a slow rate of shortening and desentitization to subsequent doses was observed. As with histamine the best results were obtained 4 to 6 weeks after denervation. Mepyramine, $5 \times 10^{-9}$ M, prevented the response and no response to antigen was seen in innervated preparations or in denervated preparations from non-sensitized animals.

## Discussion

The usual preparation used in studies of denervated skeletal muscle is the rat diaphragm, which is denervated by cutting the phrenic nerve in the neck. The denervated diaphragm is not an ideal preparation, particularly in the guinea-pig. Not only is the actual operation technically more difficult in the guinea-pig than in the rat, but having a diaphragm quiescent on one side increases the possibility of respiratory infections in an animal which is already very susceptible to such infections. Another important factor is that the setting up of a diaphragm preparation necessitates cutting through muscle fibres, which decreases the viability of the preparation. The cremaster muscle on the other hand forms a neat sac which can be dissected out with little damage to the muscle. Grant (1966) in a study on the effect of denervation on skeletal muscle blood vessels, used the cremaster muscle of the rat and reported that section of the genito-femoral nerve caused most of the myelinated nerve fibres to the muscle to degenerate, though many of the perivascular nerve fibres were left intact. We found that section of the genito-femoral nerve generally produced gradual atrophy of the cremaster muscle indicating that this nerve provides the main innervation for the muscle. The genito-femoral nerve can easily be isolated and divided and as it innervates little else besides the cremaster muscle and a small area of skin, its section produces little interference with the animal in comparison with the cutting of the phrenic nerve.

It should be stated that when using isotonic recording with the cremaster preparation the actual change of length of the muscle is much less than the change seen in a smooth muscle preparation such as guinea-pig ileum or uterus. The maximum contraction produced by KCl 0.1 M represented about 50% change in actual muscle length and the maximum contraction to ACh in denervated preparations was between 25% and 35% of the muscle length. It was nevertheless possible to obtain surprisingly consistent graded responses to drugs within quite a narrow range of change of muscle length. With isometric recording on the other hand quite substantial increases of tension could be obtained not only with KCl but also with ACh.

### *The response of the innervated muscle to acetylcholine*

One unexpected finding was that the innervated preparations exposed to ACh gave slow dose-related contractile responses. These responses were competitively antagonized by tubocurarine but not atropine, and values obtained for the affinity constants of the antagonists indicated that the receptors were mainly if not entirely nicotinic. This implies that the muscle involved is almost certainly skeletal muscle. Ninomiya (1975) had suggested that some of the slow pharmacological responses which he reported were due to the presence of smooth muscle, possibly that associated with blood vessels. We feel that the ACh responses obtained in the present study (involving contractures of up to 5 g, competitively antagonized by (+)-tubocurarine) could not be attributed to the presence of smooth muscle.

Slow contractile responses with ACh are not usually seen with mammalian skeletal muscle preparations. One could postulate that the cremaster muscle gave sustained contractions with ACh, instead of twitches, because the distribution of receptors is different in cremaster muscle from that in most other mammalian skeletal muscle and more like that in the classical frog rectus preparation and some avian muscles (Hess, 1970). There is evidence that there are some skeletal muscles in mammals, for example, the extraocular muscles in many species, which contain muscle fibres that have multiple nerve terminals and which, after a nerve impulse or the application of ACh undergo a slow contracture rather than a twitch (see Hess, 1970). It is possible that the guinea-pig cremaster contains a component of this slow tonic skeletal muscle. Another explanation is that the thinness of the muscle and the open 'basket-work'-like nature of the arrangement of the muscle fibres allows more rapid diffusion of the ACh so that it is able to reach a high concentration at the end plates of individual muscle fibres before there is marked electrical accommodation, or hydrolysis. Preliminary microscopic observations did not support the view that a substantial part of the tissue was smooth muscle. This is in accord with the observations of Grant (1966) and Majno, Palade & Schoefl (1961) who did detailed histological studies on rat cremaster muscle and described the muscle as predominantly striated in nature. However, it is still possible that some smooth muscle could be present, though it is unlikely that it is implicated in the responses to cholinomimetic agents described in this study.

### *The response of the denervated muscle to acetylcholine*

On the basis of the results obtained in the present study the ACh receptors in the denervated preparations were also nicotinic. In this respect our results with this muscle differ from those obtained with the denervated anterior gracilis muscle of the rat *in situ*. Turkanis (1969) using this latter preparation found that equal doses of atropine and (+)-tubocurarine produced equivalent effects in that they necessitated the same increase in the amount of ACh required to produce a given amount of depolarization of the muscle fibre membrane. However, Dale & Gaddum (1930) and Beránek & Vyskočil (1967) using *in vitro* preparations found that the concentration of atropine had to be 300 times greater than that of tubocurarine to produce the same effect. Our results with the denervated cremaster muscle are in accord with these latter observations.

### *The effect of anticholinesterases*

One other perhaps rather unexpected finding with this muscle was that the ACh response in denervated muscle showed some potentiation in the presence of anticholinesterases. In fact the potentiation was similar to that seen in innervated muscle. One explanation is that the muscle had not been completely denervated and that some cholinesterase was still present in association with a few remaining nerve fibres (Grant, 1966). It would be of interest to examine the distribution of cholinesterase in this muscle before and after denervation.

### *The response to histamine*

It was reported by Alonso-de Florida *et al.* (1965) that denervated guinea-pig diaphragm responded to histamine. Ninomiya (1975) claimed that histamine increased basal tension and generated spontaneous contractions in a cut section of innervated cremaster muscle—the tip. In the present study we were unable to obtain contractions to histamine in whole innervated cremaster muscle, but a reasonable dose-response curve to histamine could be obtained with the denervated preparation. The histamine responses could be antagonized by the $H_1$-receptor antagonist, mepyramine, but not by the $H_2$-receptor antagonist metiamide. The affinity constant obtained for mepyramine fits in with those obtained from other tissues with $H_1$-receptors. Similarly selective effects with these two types of antagonists were also obtained in denervated guinea-pig diaphragm by Dale & Evinç (unpublished results). If the histamine response of denervated guinea-pig skeletal muscle is due to the appearance or uncovering of receptors for histamine, it would seem that these receptors are $H_1$-receptors.

### *Spontaneous contractions*

Ninomiya (1975) reported spontaneous contractions in cremaster preparations. We did not obtain spontaneous contractions in any preparations in which isotonic recording methods were employed. In a few preparations in which isometric-recording was used, spontaneous contractions of 100–250 mg were seen towards the end of a long experiment. These small fluctuations in basal tension could well have been due to smooth muscle, but we did not find that either histamine or ACh increased them.

The difference between our results and Ninomiya's could possibly be due to strain differences in guinea-pigs or to the fact that he used a cut section of the muscle, the tip, whereas all our experiments were performed on whole intact muscles.

### *The response of the denervated preparation to catecholamines*

Many studies have reported that denervated skeletal muscle develops sensitivity to catecholamines (Ellis, 1959; Bhoola & Schachter, 1961; Bowman & Raper, 1965) though Turkanis (1969) reported that topically applied catecholamines had no effect on the denervated anterior gracilis muscle of the rat. In the present study adrenaline and noradrenaline both produced sustained contractures. These responses also occurred in the presence of atropine and (+)-tubocurarine, so, as was concluded by Bowman & Raper (1965), cholinergic mechanisms are not involved. Isoprenaline produced virtually no response except in very high concentration. These findings suggest that if catecholamine receptors are present they are possibly $\alpha$-receptors. Paterson (1963) using rat hemidiaphragm concluded that $\alpha$-receptors were present in denervated skeletal muscle, whereas Bowman & Raper (1965) using the denervated tibialis anterior of the cat concluded that $\beta$-receptors were present.

The adrenaline response reported by Ninomiya (1975) in innervated muscle could be due either to the smooth muscle which he postulates is present, or to slow tonic skeletal muscle, which has also been reported to give contractures with adrenaline (Bowman & Nott, 1969).

### *The anaphylactic response of denervated muscle*

The denervated cremaster muscle from guinea-pigs sensitized with ovalbumin gave typical Schultz-Dale responses when exposed to the antigen *in vitro*. The peak time for the reaction coincided with the peak time for the histamine response, 6 weeks after denervation, and like the histamine response it could be antagonized by mepyramine. A similar anaphylactic response has been reported by Alonso-de Florida *et al.* (1965) and Alonso-de Florida, del Castillo, García & Gijón (1968) and attributed by them to direct antigen-antibody stimulation of muscle contraction. It was pointed out by Dale & Ziletti (1970) that most evidence supports the view that the anaphylactic contraction of smooth muscle is mainly due to mediators released from mast cells and that this could well be the case for the denervated skeletal muscle preparation used by Alonso-de Florida *et al.* (1965), because the tendinous part of guinea-pig diaphragm is certainly very rich in mast cells. One would have to postulate that if, in the cremaster muscle, mediator-release was indeed the basis of the anaphylactic reaction, the main mediator involved was probably histamine, because other substances which are believed to be implicated in the anaphylactic contraction of smooth muscle are not active in denervated cremaster muscle. It would be of interest to examine the histamine content and mast cell distribution of the denervated cremaster muscle.

We are grateful to Professor H.O. Schild and Dr D.H. Jenkinson for helpful criticism and to Miss C. Morris and Mr R. Muid for technical assistance.

### References

ALONSO-de FLORIDA, F., del CASTILLO, J., GONZALEZ, C.G. & SANCHEZ, V. (1965). On the pharmacological and anaphylactic responsiveness of denervated skeletal muscle of the guinea-pig. *Br. J. Pharmac. Chemother.*, **25**, 610–620.

ALONSO-de FLORIDA, F., del CASTILLO, J., GARCÍA, X. & GIJÓN, E. (1968). Mechanism of the Schultz-Dale reaction in the denervated diaphragmatic muscle of the guinea pig. *J. gen. Physiol.*, **51**, 677–693.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48–58.

ASH, A.S.F. & SCHILD, H.O. (1966). Receptors mediating some actions of histamine. *Br. J. Pharmac. Chemother.*, **27**, 427–439.

BARLOW, R.B. (1964). *Introduction to chemical pharmacology*. 2nd ed. p. 107. London: Methuen & Co. Ltd.

BERÁNEK, R. & VYSKOČIL, F. (1967). The action of tubocurarine and atropine on the normal and denervated rat diaphragm. *J. Physiol., Lond.*, **188**, 53–66.

BHOOLA, K.D. & SCHACHTER, M. (1961). Contracture of the denervated rat diaphragm by adrenaline. *J. Physiol., Lond.*, **157**, 20–21.

BOWMAN, W.C. & NOTT, M.W. (1969). Actions of sympathomimetic amines and their antagonists on skeletal muscle. *Pharmac. Rev.*, **21**, 27–72.

BOWMAN, W.C. & RAPER, C. (1965). The effect of sympathomimetic amines on chronically denervated skeletal muscles. *Br. J. Pharmac., Chemother.*, **24**, 98–109.

DALE, H.H. & GADDUM, J.H. (1930). Reactions of denervated voluntary muscle, and their bearing on the mode of action of parasympathetic and related nerves. *J. Physiol., Lond.*, **70**, 109–144.

DALE, M.M. & ZILETTI, L. (1970). The Schultz-Dale response of the longitudinal muscle strip preparation of guinea-pig ileum. *Br. J. Pharmac.*, **39**, 542–555.

ELLIS, S. (1959). Relation of biochemical effects of epinephrine to its muscular effects. *Pharmac. Rev.*, **11**, 469–479.

ELMQVIST, D. & THESLEFF, S. (1960). A study of acetylcholine induced contractures in denervated mammalian muscle. *Acta Pharmac. tox.*, **17**, 84–93.

GRANT, R.T. (1966). The effects of denervation of skeletal muscle blood vessels (rat cremaster). *J. Anat.*, **100**, 305–316.

HESS, A. (1970). Vertebrate slow muscle fibers. *Physiol. Rev.*, **50**, 40–62.

LANGLEY, J.N. & KATO, T. (1915). The physiological action of physostigmine and its action on denervated skeletal muscle. *J. Physiol., Lond.*, **49**, 410–431.

LU, T. (1970). Affinity of curare-like compounds and their potency in blocking neuromuscular transmission. *J. Pharmac. exp. Ther.*, **174**, 560–566.

MAJNO, G., PALADE, G.E. & SCHOEFL, G.I. (1961). Studies on Inflammation II. The site of action of histamine and serotonin along the vascular tree: a topographic study. *J. biophys. biochem. Cytol.*, **11**, 607–626.

NINOMIYA, J.G. (1975). Some pharmacological properties of the cremaster muscle of the guinea-pig. *Br. J. Pharmac.*, **55**, 487–496.

PATERSON, G. (1963). The dual action of adrenaline on denervated skeletal muscle. *Biochem. Pharmac.*, **12**, Suppl., p. 85.

SCHILD, H.O. (1949). $pA_x$ and competitive drug antagonism. *Br. J. Pharmac. Chemother.*, **4**, 277–280.

TURKANIS, S.A. (1969). Some properties of the denervated anterior gracilis muscle of the rat. *Br. J. Pharmac. Chemother.*, **37**, 414–424.

*(Received February 12, 1976.*
*Revised April 18, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 239–245

# THE ACTION OF MICRO-ELECTROPHORETICALLY APPLIED L–3,4-DIHYDROXYPHENYLALANINE (DOPA) ON SINGLE CORTICAL NEURONES

P. BEVAN, C.M. BRADSHAW & E. SZABADI
Department of Psychiatry, University of Manchester, Stopford Building, Oxford Road, Manchester, M13 9PT

1 The technique of microelectrophoresis was used in order to compare the actions of L-3,4-dihydroxyphenylalanine (DOPA) and noradrenaline on single neurones in the cerebral cortices of cats and rats.

2 DOPA could both excite and depress cortical neurones. Cells excited by DOPA were also excited by noradrenaline and cells depressed by DOPA were also depressed by noradrenaline.

3 In the case of both excitatory and depressant responses, DOPA appeared to be less potent than noradrenaline.

4 Responses to DOPA and noradrenaline could be antagonized by phentolamine and propranolol. Responses to acetylcholine were not affected.

5 Responses to acetylcholine, but not responses to DOPA, were antagonized by atropine.

6 The results indicate that locally applied DOPA may mimic the actions of noradrenaline on cortical neurones. Possible mechanisms for these effects of DOPA are discussed.

## Introduction

L-3,4-Dihydroxyphenylalanine (DOPA) is widely used in the treatment of Parkinson's disease. DOPA is the metabolic precursor of dopamine, and it is generally assumed that DOPA exerts its therapeutic effect via the synthesis and release of dopamine in the caudate nucleus (Hornykiewicz, 1974). However, DOPA is also a precursor of noradrenaline (NA), and it has been suggested that in structures receiving a NA innervation, exogenously administered DOPA may cause the release of NA from presynaptic terminals and thus mimic the actions of NA on post-synaptic cells (Andén, Carlsson & Häggendal, 1969). In the experiments described here we compared the effects of microelectrophoretically applied DOPA and NA on single neurones in the cerebral cortex, since this structure is known to be innervated by NA-containing neurones (Fuxe, 1965).

Some of these results have been reported to the British Pharmacological Society (Bevan, Bradshaw & Szabadi, 1975a).

## Methods

The experiments were conducted on adult cats of either sex (2–3.5 kg) and on male albino Wistar rats (250–300 grams). The animals were anaesthetized with halothane (0.5–1.2%). Our methods for the surgical preparation of the animals and the manufacture of six-barrelled glass micropipettes for extracellular recording and microelectrophoretic drug application, have been described previously (Bradshaw, Roberts & Szabadi, 1973a; Bradshaw, Szabadi & Roberts, 1973b; Bevan, Bradshaw & Szabadi, 1975b).

Micropipettes having tip diameters of 3–5 μm were used. Two barrels of each micropipette contained 4 M NaCl, one barrel for recording action potentials, the other for use in current balancing (Roberts & Straughan, 1967). The remaining barrels contained drug solutions. The following drug solutions were used: L-3,4-dihydroxyphenylalanine methylester hydrochloride (0.05 M, pH 5.0), (−)-noradrenaline bitartrate (0.05 M, pH 3.0–3.5), acetylcholine chloride (0.05 M, pH 3.5–4.0), phentolamine mesylate (0.01 M, pH 4.5–5.5), propranolol hydrochloride (0.01 M, pH 4.5–5.5) and atropine sulphate (0.01 M, pH 5.5–6.0).

Only spontaneously active neurones were studied in these experiments. All the drugs were applied by microelectrophoresis. When a suitable unit was encountered, the agonists were applied in a regular cycle. Between successive drug applications retaining

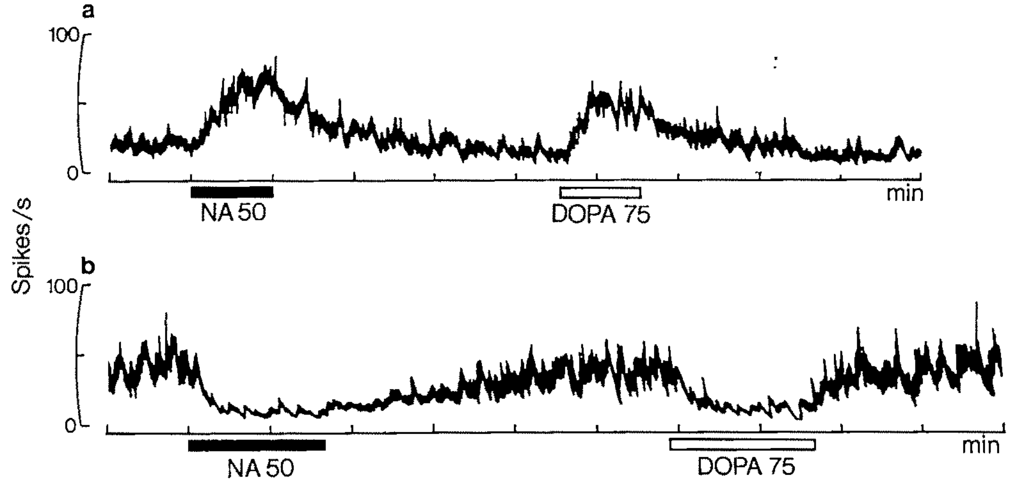


**Figure 1** Correlation between effects of L-3,4-dihydroxyphenylalanine (DOPA) and noradrenaline (NA). Ratemeter recordings of the firing rates of two cortical neurones in the rat (a and b). Ordinates: firing rate (spikes/s); abscissae: running time (min). Horizontal bars indicate microelectrophoretic drug applications; numbers refer to intensities of ejecting currents (nA). (a) A cell which was excited by both DOPA and NA; (b) a cell which was depressed by both DOPA and NA.

currents of −10 nA were passed. Intervals between successive applications of the same agonist were kept constant in order to standardize the effects of the retaining current upon drug release during the ejection periods (Bradshaw *et al.*, 1973a; 1973b). The sizes of the neuronal responses to the agonists were expressed as the total number of action potentials produced in response to each drug application ('total spike number'). The total spike number was calculated by measuring the number of spikes generated between the onset of the drug application and the recovery of the baseline firing rate, and subtracting the number of spikes generated during an equivalent control period (Bradshaw *et al.*, 1973b; Bevan, Bradshaw, Roberts & Szabadi, 1974).

The effects of antagonists were evaluated in the following way. When stable responses to the agonists had been obtained, the antagonist was applied continuously, either by removal of the retaining current (thus allowing the drug to diffuse out from the micropipette) or by the passage of a weak ejecting current (5 to 10 A), and the time-course of the developing antagonism was followed. If necessary, the intensity of the ejecting current was increased until antagonism was observed. Then the application of the antagonist was terminated and the time-course of recovery was followed. The response to an agonist was regarded as antagonized if there was at least a 50% reduction in the total spike number (Bevan *et al.*, 1974).

## Results

### *Agonistic effect of DOPA*

*Responses of cortical neurones to DOPA.* Both excitatory and depressant responses to DOPA were observed in these experiments. The effect of DOPA (25–150 nA, applied for 25–100 s) was tested on 51 neurones in the rat; 44 were excited and 7 were depressed by DOPA. Eleven neurones were studied in the cat; of these 10 were excited and one depressed by DOPA. The predominance of excitatory responses in both species is statistically significant (binomial test, $P<0.01$ in both cases).

*Direction of responses to DOPA and noradrenaline.* Responses to both DOPA and NA were obtained from 40 cells (cat 10 cells; rat 30 cells). All of these cells responded in the same direction to the two drugs, cells excited by DOPA being also excited by NA and cells depressed by DOPA being also depressed by NA (Figure 1). This positive correlation between the effects of the two drugs is statistically significant ($\chi^2$ test, $P<0.001$).

*Comparison of apparent potencies of DOPA and noradrenaline.* In 34 of the 40 cells on which the effects of DOPA and NA were compared, DOPA appeared to be less potent than NA (binomial test, $P<0.01$). When DOPA and NA were applied to the

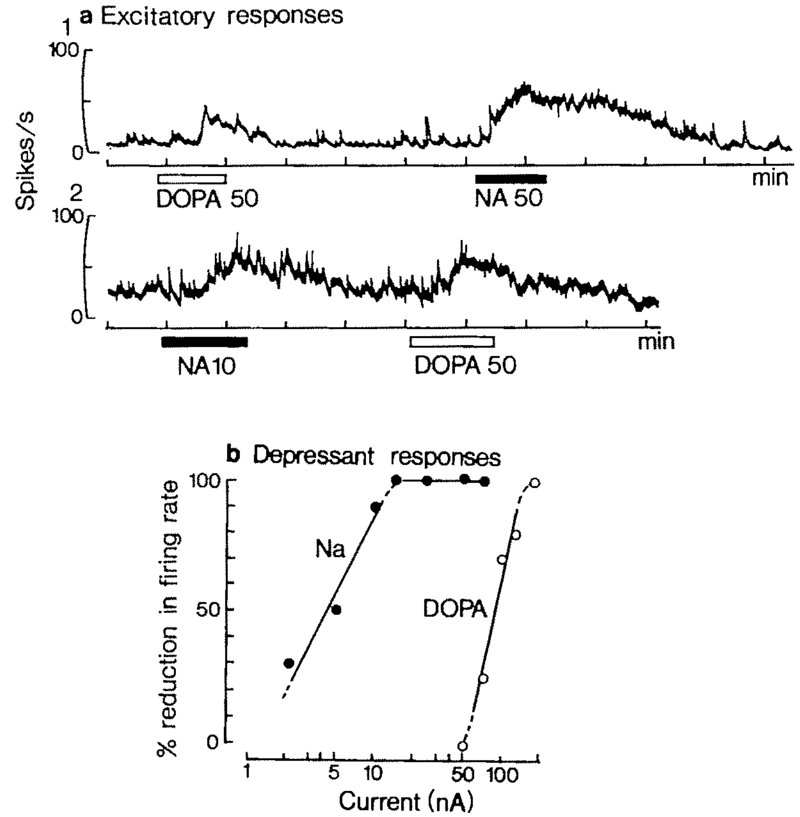


**Figure 2** Comparison of apparent potencies of noradrenaline (NA) and L-3,4-dihydroxyphenylalanine (DOPA). (a) Excitatory responses. Ratemeter recordings of firing rates of single cortical neurones in cat (trace 1) and rat (trace 2). (1): DOPA evoked a smaller response than NA when both drugs were applied with identical ejecting pulses; (2) in order to evoke approximately equivalent responses, a higher current was needed to apply DOPA than NA. (b) Depressant responses. Current-response curves for suppression of firing rate, measured at equilibrium, plotted against intensity of ejecting current, on a log scale. Data were obtained from a single cortical neurone in the rat. Each point represents one individual response. DOPA was apparently less potent than NA; the equipotent current ratio at 50% response was 18.

same cell with identical ejecting pulses the size of the response (see Methods section) evoked by NA was 1.8–3.4 times greater (inter-quartile range) than the response evoked by DOPA. In order to obtain approximately equivalent responses to the two drugs, the current needed to apply DOPA was 2.0–3.0 times greater (inter-quartile range) than that needed to apply NA. The lower potency of DOPA than NA was apparent in the case of both excitatory and depressant responses. Examples are shown in Figure 2.

*Comparison of latencies to onset of responses to DOPA and noradrenaline.* The latencies to onset of responses to DOPA and NA were compared in 12 cells to which the two drugs were applied with identical ejecting currents. The latencies of responses to DOPA were 1.7–2.3 times greater (inter-quartile range) than those of responses to NA.

### *Effects of antagonists on responses to DOPA*

In these experiments the effects of the antagonists on responses to DOPA were compared with their effects on responses to NA and acetylcholine (ACh).

*Phentolamine.* The effects of phentolamine were studied on 12 cells excited by DOPA (cat: 6 cells; rat: 6 cells). In all the cells tested, phentolamine (0–40 nA, applied for 10–60 min) reversibly antagonized excitatory responses to DOPA; excitatory responses to NA were also antagonized. These effects of phentolamine occurred at times when responses to ACh were not affected (see Figure 3). However, more prolonged applications of phentolamine were sometimes accompanied by loss of spike amplitude and reduced sensitivity to ACh.

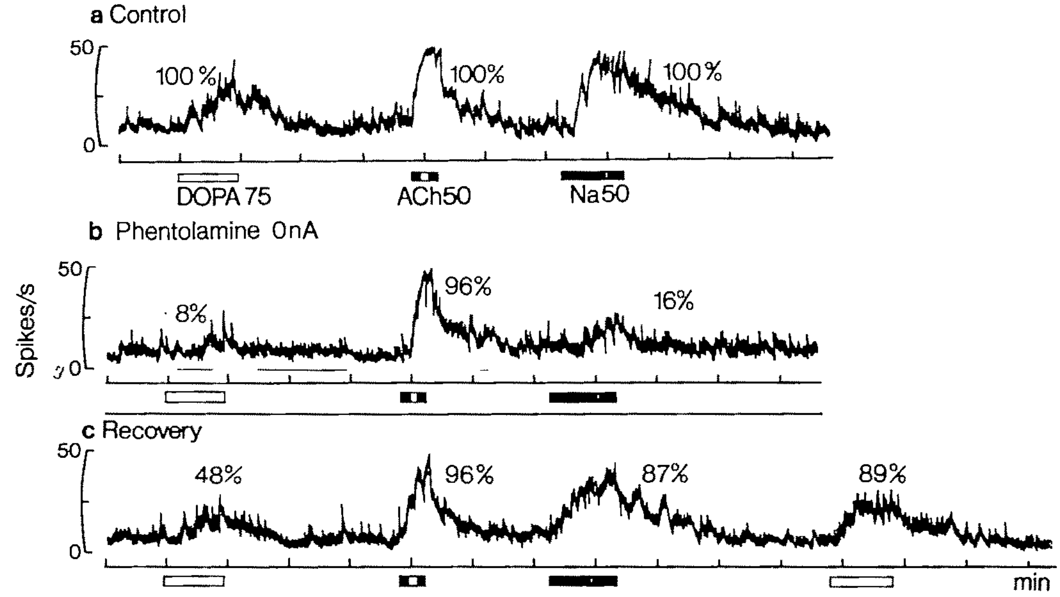


**Figure 3** Effect of phentolamine on excitatory responses to L-3,4-dihydroxyphenylalanine (DOPA), noradrenaline (NA) and acetylcholine (ACh). Ratemeter recording of firing rate of single cortical neurone in the rat. Figures above the traces indicate total spike numbers (%) taking the sizes of the control responses to each agonist as 100%. (a) Control responses to the agonists; (b) responses to the agonists during the continuous application of phentolamine. Phentolamine was applied by removal of the retaining current and at the start of trace (b) had been applied continuously for 21 minutes. The responses to NA and DOPA, but not the response to ACh, were antagonized. (c) Recovery of responses, 16 min after the application of phentolamine had been terminated.

In two cells in the rat, phentolamine failed to antagonize depressant responses to DOPA and NA.

*Propranolol.* The effects of propranolol on excitatory responses to DOPA, NA and ACh were tested on 11 cells in the rat. In 8 of these cells propranolol (0–10 nA, applied for 10–40 min) reversibly antagonized the responses to DOPA and NA without affecting responses to ACh (see Figure 4). In the remaining 3 cells, propranolol produced a reduction in spike amplitude which precluded successful drug interaction studies.

*Atropine.* The effects of atropine on excitatory responses to DOPA and ACh were tested on 6 cells in the rat. In all the cells tested, atropine (0–10 nA, applied for 10–30 min) reversibly antagonized the responses to ACh with little effect on responses to DOPA. An example of a cell on which the effects of both atropine and propranolol were studied is shown in Figure 5. On one cell atropine reversibly abolished

**Figure 4** Effect of propranolol on excitatory responses to L-3,4-dihydroxyphenylalanine (DOPA), noradrenaline (NA) and acetylcholine (ACh). Ratemeter recording of firing rate of a single cortical neurone in the rat (as in Figure 3). (a) Control responses to the agonists; (b) responses to the agonists during the continuous application of propranolol. At the start of trace (b) propranolol (10 nA) had been applied continuously for 11 minutes. The responses to NA and DOPA, but not the response to ACh, were antagonized. (c) Recovery of responses, 6.5 min after the application of propranolol had been terminated.

**Figure 5** Comparison of effects of propranolol and atropine on excitatory responses to L-3,4-dihydroxyphenylalanine (DOPA) and acetylcholine (ACh). Ratemeter recording of firing rate of single cortical neurone in the rat (as in Figure 3): (a) and (b) are from two studies conducted on the same neurone. (a) Effect of propranolol: (i) control responses to DOPA and ACh; (ii) responses to the agonists during the continuous application of propranolol. At the start of trace (ii), propranolol (10 nA) had been applied continuously for 7.5 minutes. The response to DOPA, but not the response to ACh, was antagonized. (iii) Recovery of responses, 8 min after the application of propranolol had been terminated. (b) Effect of atropine: (i) control responses to DOPA and ACh; (ii) responses to the agonists during the continuous application of atropine. At the start of trace (ii), atropine (10 nA) had been applied for 8 minutes. The response to ACh was antagonized, but the response to DOPA was not greatly affected. (iii) Recovery of the responses 12 min after the application of atropine had been terminated.

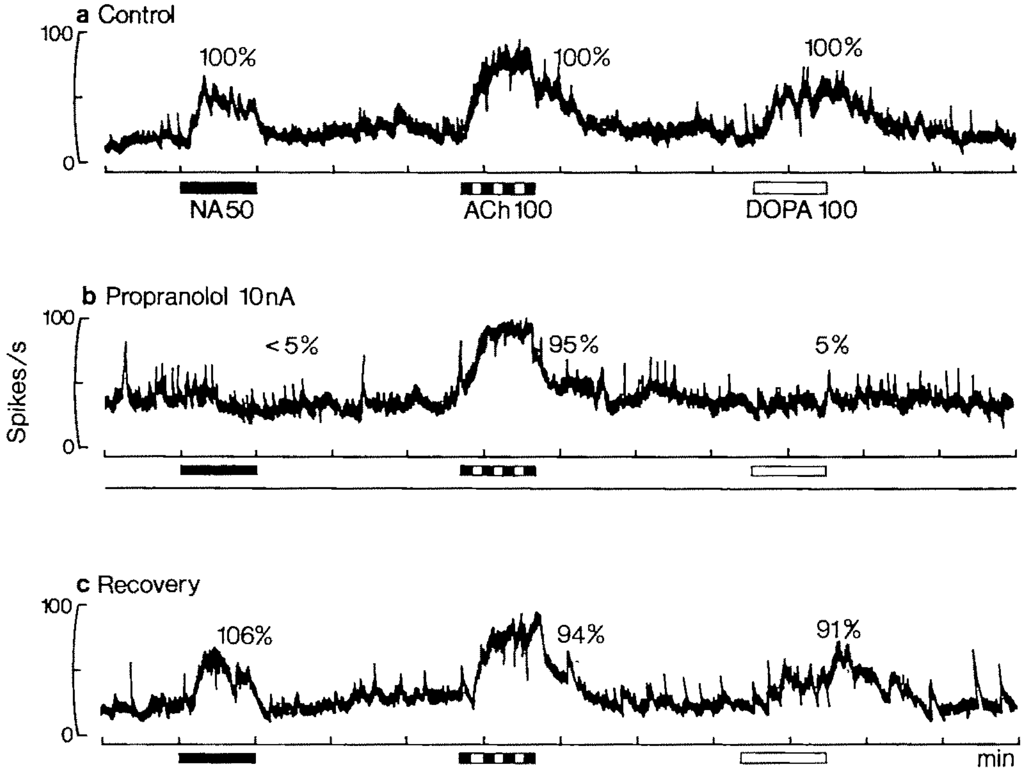


**Figure 4**

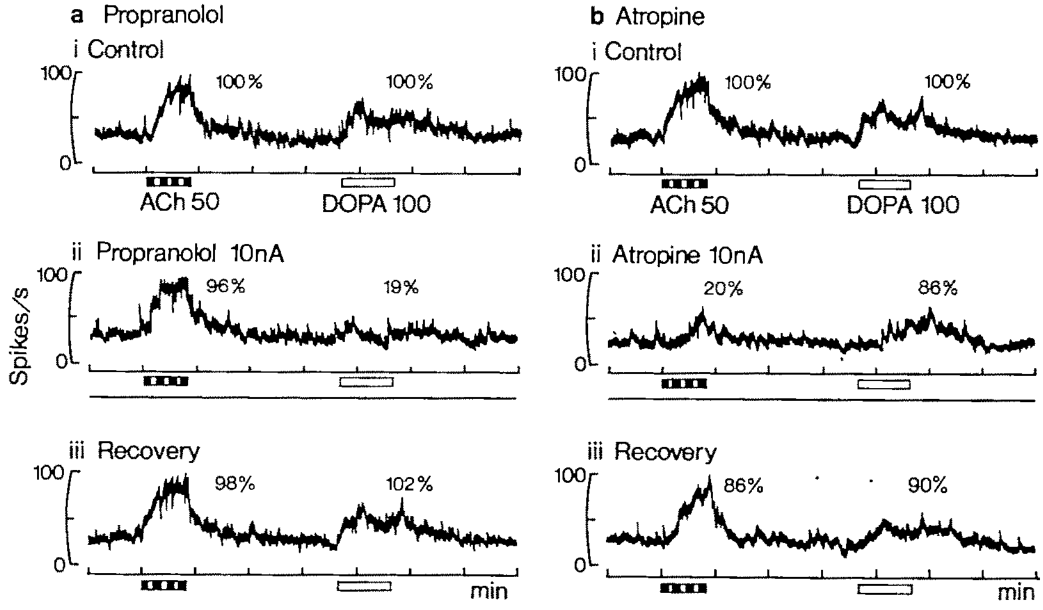


**Figure 5**

the excitatory effects of ACh without affecting depressant responses to DOPA.

## Discussion

The results described here indicate that the effects of DOPA on single cortical neurones are very similar to those of NA. Firstly, it was found that cells invariably responded in the same direction to the two drugs. This contrasts with much lower correlations between the effects of NA and ACh, or NA and 5-hydroxytryptamine on cortical neurones (Johnson, Roberts, Sobieszek & Straughan, 1969; Bradshaw, Roberts & Szabadi, 1971; Bevan *et al.*, 1974). Secondly, the excitatory effects of both DOPA and NA could be abolished by phentolamine and propranolol when responses to ACh remained unaffected, while responses to DOPA were not affected by atropine when responses to ACh were completely abolished. The selectivity of these antagonists with respect to NA and ACh in our experiments is in agreement with the observations of Johnson *et al.* (1969). The present finding of a close correlation between the effects of DOPA and NA seems to be in contrast with the observations of Krnjević & Phillis (1963) who reported that DOPA had a weak excitatory action on cortical neurones while NA was predominantly depressant. However, these authors did not report within-cell comparisons of the effects of the two drugs.

A possible explanation for the agonistic effects of DOPA seen in our experiments is the release of NA from noradrenergic nerve terminals. There is evidence that such a mechanism underlies the facilitatory effects of DOPA on spinal reflexes (Andén *et al.*, 1969; Andén, Engel & Rubenson, 1972a) and the stimulant effects of DOPA on locomotor activity (Corrodi, Fuxe, Ljungdahl & Ögren, 1970). Since these actions of DOPA coincide with raised levels of NA in the CNS, and can be blocked by drugs which inhibit the synthesis of NA from DOPA, it is unlikely that DOPA exerts a direct action of its own in these test systems. However, in the present experiments, the possibility cannot be excluded that DOPA acted directly on post-synaptic NA receptors. The observation that the latency to onset of responses to DOPA was greater than that of responses to NA might be taken as evidence in favour of an indirect action of DOPA; however the dependence of response latency upon physical factors such as transport number, diffusion coefficient and the parameters of the ejecting and retaining currents (Bradshaw *et al.*, 1973a, b; Szabadi & Bradshaw, 1974) makes such observations very difficult to interpret.

An alternative possibility is that agonistic actions of DOPA were mediated by dopamine rather than NA. Recent evidence suggests that there are dopamine-containing nerve terminals in the cerebral cortex (Thierry, Hirsch, Tassin, Blanc & Glowinski, 1974; Fuxe, Hökfelt, Johansson, Jonsson, Lidbrink & Ljungdahl, 1974) and that dopamine can be synthesized in these terminals from systemically administered DOPA (Hökfelt, Ljungdahl, Fuxe & Johansson, 1974). Moreover, DOPA may be decarboxylated in 5-hydroxytryptamine-containing nerve terminals, with the subsequent release of dopamine (Butcher, Engel & Fuxe, 1970). If, in the present experiments, DOPA acted either directly or indirectly on dopamine receptors, then the antagonistic effects of phentolamine and propranolol on responses to DOPA and NA would indicate that dopamine receptors are highly similar to NA receptors on cortical neurones.

Finally, some comment is needed about the apparently lower potency of DOPA than NA seen in our experiments. Such a difference in potency might reflect physical factors, such as differences between the transport numbers and diffusion coefficients of the two drugs (Szabadi & Bradshaw, 1974). However, the lower potency of DOPA might also be due to biological factors, such as the extra-neuronal accumulation of DOPA (Butcher *et al.*, 1970; Andén *et al.*, 1972b), the retention of newly synthesized catecholamines in presynaptic stores after DOPA administration (Butcher *et al.*, 1970), or, if the primary action of DOPA is post-synaptic, differences between the affinities and/or intrinsic activities of the two drugs at NA receptors.

This work was supported by the Mental Health Trust and Research Fund. We are grateful to Mr R. Lamb for technical assistance.

## References

ANDÉN, N.-E., CARLSSON, A. & HÄGGENDAL, J. (1969). Adrenergic mechanisms. *Ann. Rev. Pharmac.*, **9**, 119–134.

ANDÉN, N.-E., ENGEL, J. & RUBENSON, A. (1972a). Mode of action of L-DOPA on central noradrenaline mechanisms. *Naunyn-Schmiedebergs Arch. Pharmac.*, **273**, 1–10.

ANDÉN, N.-E., ENGEL, J. & RUBENSON, A. (1972b). Central decarboxylation and uptake of L-DOPA. *Naunyn-Schmiedebergs Arch. Pharmac.*, **273**, 11–26.

BEVAN, P., BRADSHAW, C.M., ROBERTS, M.H.T. & SZABADI, E. (1974). The effect of microelectrophoretically applied mescaline on cortical neurones. *Neuropharmacology*, **13**, 1033–1045.

BEVAN, P., BRADSHAW, C.M. & SZABADI, E. (1975a). Comparison of the effects of DOPA and noradrenaline on single cortical neurones. *Br. J. Pharmac.*, **55**, 301–302P.

BEVAN, P., BRADSHAW, C.M. & SZABADI, E. (1975b). Effects of iprindole on responses of single cortical and caudate neurones to monoamines and acetylcholine. *Br. J. Pharmac.*, **55**, 17–25.

BRADSHAW, C.M., ROBERTS, M.H.T. & SZABADI, E. (1971). Effect of mescaline on single cortical neurones. *Br. J. Pharmac.*, **43**, 871–873.

BRADSHAW, C.M., ROBERTS, M.H.T. & SZABADI, E. (1973a). Kinetics of the release of noradrenaline from micropipettes: interaction between ejecting and retaining currents. *Br. J. Pharmac.*, **49**, 667–677.

BRADSHAW, C.M., SZABADI, E. & ROBERTS, M.H.T. (1973b). The reflection of ejecting and retaining currents in the time-course of neuronal responses to microelectrophoretically applied drugs. *J. Pharm. Pharmac.*, **25**, 513–520.

BUTCHER, L., ENGEL, J. & FUXE, K. (1970). L-DOPA induced changes in central monoamine neurones after peripheral decarboxylase inhibition. *J. Pharm. Pharmac.*, **22**, 313–315.

CORRODI, H., FUXE, K., LJUNGDAHL, A. & ÖGREN, S.-O. (1970). Studies on the action of some psychoactive drugs on central noradrenaline neurones after inhibition of dopamine-$\beta$-hydroxylase. *Brain Res.*, **24**, 451–470.

FUXE, K. (1965). Evidence for the existence of monoamine neurones in the central nervous system. IV. Distribution of monoamine nerve terminals in the central nervous system. *Acta physiol. scand.*, **64**, Suppl. 247.

FUXE, K., HÖKFELT, T., JOHANSSON, O., JONSSON, G., LIDBRINK, P. & LJUNGDAHL, Å. (1974). The origin of the dopamine nerve terminals in limbic and frontal cortex. *Brain Res.*, **82**, 349–355.

HÖKFELT, T., LJUNGDAHL, Å., FUXE, K. & JOHANSSON, O. (1974). Dopamine nerve terminals in the rat limbic cortex: aspects of the dopamine hypothesis of schizophrenia. *Science*, **184**, 177–179.

HORNYKIEWICZ, O. (1974). The mechanisms of action of L-DOPA in Parkinson's disease. *Life Sci.*, **15**, 1249–1259.

JOHNSON, E.S., ROBERTS, M.H.T., SOBIESZEK, A. & STRAUGHAN, D.W. (1969). Noradrenaline sensitive cells in cat cerebral cortex. *Int. J. Neuropharmac.*, **8**, 549–566.

KRNJEVIĆ, K. & PHILLIS, J.W. (1963). Actions of certain amines on cerebral cortical neurones. *Br. J. Pharmac. Chemother.*, **20**, 471–490.

ROBERTS, M.H.T. & STRAUGHAN, D.W. (1967). Excitation and depression of cortical neurones by 5-hydroxytryptamine. *J. Physiol., Lond.*, **193**, 269–294.

SZABADI, E. & BRADSHAW, C.M. (1974). The role of physical and biological factors in determining the time-course of neuronal responses. *Neuropharmacology*, **13**, 537–545.

THIERRY, A.M., HIRSCH, J.C., TASSIN, J.P., BLANC, G. & GLOWINSKI, J. (1974). Presence of dopaminergic terminals and absence of dopaminergic cell bodies in the cerebral cortex of the cat. *Brain Res.*, **79**, 77–88.

*(Received February 26, 1976.*
*Revised April 23, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 247–252

# EFFECTS OF KETAMINE ON THE PERIPHERAL AUTONOMIC NERVOUS SYSTEM OF THE RAT

A.S. CLANACHAN[1]
Department of Anaesthesia, Royal Infirmary, Glasgow
J.C. McGRATH[2]
Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

**1** The effects of ketamine (2-(*o*-chlorophenyl) 2-methylaminocyclohexanone) (2–50 mg/kg) on the responses of the pithed rat arterial pressure, anococcygeus muscle and colon to selective stimulation of the spinal autonomic outflows were examined.

**2** Ketamine depressed the vasopressor response produced by stimulation of the lumbar sympathetic outflow in a dose-dependent manner but did not significantly affect the pressor response to intravenous noradrenaline (NA) administration.

**3** Ketamine depressed the motor responses of the anococcygeus to stimulation of the pre-ganglionic lumbar sympathetic outflow or to stimulation of post-ganglionic fibres in the sacral region in a dose-dependent manner, the response to preganglionic stimulation being relatively more sensitive to such depression. The anococcygeus response to NA was significantly potentiated with doses of ketamine of 20 mg/kg and 50 mg/kg.

**4** Ketamine depressed the motor response of the smooth muscle of the colon to stimulation of the sacral parasympathetic outflow in a dose-dependent manner and at lower doses than were required to produce an equivalent depression of the sympathetic responses in the other tissues.

**5** A comparison was made of the effects of ketamine and cocaine on the motor responses of the anococcygeus muscle *in vitro* to NA, carbachol and field stimulation. Both ketamine and cocaine produced a non-specific depression of all responses at high doses whereas cocaine but not ketamine produced a large potentiation of NA and motor nerve responses at lower doses.

**6** The results are discussed in relation to the hypothesis that ketamine might elevate blood pressure in conscious animals and man by potentiating vascular adrenergic responses.

## Introduction

Ketamine is unique among intravenous anaesthetic agents in producing an increase in blood pressure and heart rate in man and in several species of animals (Domino, Chodoff & Corssen, 1965; McCarthy, Chen, Kaump & Ensor, 1965). It has been suggested that the pressor action involves the sympathetic nervous system since it can be abolished by pretreatment with α-adrenoceptor blocking agents (Traber & Wilson, 1969), but it is not known whether the site of action is central or peripheral. Chang, Chan & Ganendran (1969) have suggested that ketamine may release catecholamines into the bloodstream in the pithed rat and that this might be responsible for its pressor action.

Recently Nedergaard (1973), and Montel, Starke, Gorlitz & Schumann (1973) have shown that ketamine can inhibit the neuronal uptake mechanism for noradrenaline (NA) *in vitro*, and can potentiate responses to both NA and sympathetic nerve stimulation both *in vitro* and with prolonged infusion of ketamine *in vivo*. The neuronal uptake process for NA has been shown to be an important factor in the physiological operation of adrenergic nerves and in the pharmacological efficacy of many drugs *in vitro* or in the central nervous system *in vivo* (Iversen, 1973) but has not until now been implicated in the peripheral actions of drugs *in vivo*. However, Muscholl (1961) has demonstrated that cocaine blocks neuronal NA uptake and potentiates pressor responses to NA in the pithed rat.

In this study, the effects of ketamine were examined on the responses of the arterial blood pressure and the anococcygeus muscle to sympathetic nerve stimulation or to intravenous NA and on the

[1] Present address: Department of Pharmacology, Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada.

[2] Present address: Institute of Physiology, University of Glasgow, Glasgow G12 8QQ.

responses of the colon to parasympathetic nerve stimulation, in the pithed rat (Gillespie, MacLaren & Pollock, 1970).

In order to compare the doses used with those used previously to block neuronal NA uptake, the effect of ketamine was also examined on the responses of the anococcygeus muscle *in vitro* to agonist drugs and to field stimulation. A preliminary account of these results has been published (Clanachan & McGrath, 1975).

## Methods

Sprague-Dawley rats (250–300 g) were used.

### *Pithed rat*

The rats were pithed under halothane by the method of Gillespie *et al.* (1970). The trachea was intubated and ventilation with 100% $O_2$ at 60 $min^{-1}$ (Harvard Instruments Rodent Ventilator) was adjusted to give an end tidal $CO_2$ of 4% (Beckman LB2 Medical Gas Analyser). Drugs were administered via a polythene cannula in the right jugular vein and arterial pressure monitored from a polythene cannula in the left carotid artery (Bell and Howell type 4-327-L221 transducer).

The tension in the anococcygeus muscle was monitored isometrically (Devices type 4151 transducer), by tying a thread around the ventral bar of the tissue via an incision in the scrotum. The tissues were kept moist by a pool of liquid paraffin (Gillespie & McGrath, 1973). Activity of the circular muscle of the colon was monitored by placing a small balloon inside and measuring pressure changes with a Bell and Howell (type 4-327-L221) transducer (Gillespie *et al.*, 1970). Rectal temperature was maintained at 37°C by warming the animal with a tungsten lamp. Anococcygeus tension, colon pressure, heart rate and arterial pressure were displayed on a Devices MX4 recorder.

Stimulation of the sympathetic outflow was by means of the unshielded 10 mm long stainless steel tip of the pithing rod and a silver indifferent electrode inserted under the skin (Devices isolated stimulator, supramaximal voltage, 1 ms pulses, 10 Hz, 10 s periods every 5 minutes). Stimulation at L1-2 produced pressor responses due to stimulation of the preganglionic sympathetic outflow to the vascular bed (Gillespie *et al.*, 1970), and contraction of anococcygeus muscles due to stimulation of the preganglionic sympathetic outflow to this latter tissue (Gillespie & McGrath, 1973). Stimulation at S1-2 produced no pressor response but contracted the anococcygeus muscles due to stimulation of postganglionic fibres to this tissue (Gillespie & McGrath, 1973), and contracted the circular muscle of the colon due to simultaneous stimulation of the preganglionic sacral parasympathetic outflow (Gillespie *et al.*, 1970). Contraction of skeletal muscle was prevented by administration of pancuronium (1 mg/kg).

Standard, reproducible, submaximal responses were obtained by stimulation of the appropriate region of the spinal canal and by the administration of NA (i.v.) at 10 min intervals. Doses of ketamine were then administered intravenously 2 min before a stimulation period or NA dose was due. At least 30 min was allowed between doses of ketamine for return of responses to control levels. Each response following ketamine was expressed as a percentage of the control response preceding ketamine.

### *Anococcygeus* in vitro

Rats were killed by a blow on the head, and exsanguination. Isolated anococcygeus muscles were studied *in vitro* by the method of Gillespie (1972); the muscles were placed in 10 ml baths containing Krebs solution at 37°C and gassed with 95% $O_2$ and 5% $CO_2$. The muscles were threaded through platinum field stimulation electrodes and tension recorded isometrically with Grass FTO3 transducers. Tension was displayed on a Grass Model 7 polygraph. Field stimulation was applied with a Tektronix stimulator (supramaximal voltage, 1 ms pulses) and drugs added to the bath in volumes of 0.2 ml or less. Reproducible submaximal responses were obtained to field stimulation at 10 Hz for 10 s periods or to addition to the bath of NA 3 μM or carbachol 1 μM. Sufficient contact time was allowed with the agonist drugs to reach a plateau response. These parameters were chosen since they produce responses of similar magnitude; approximately 50% of the maximum response of the tissue. When each concentration of ketamine and cocaine was added to the bath, at least 5 min was allowed for equilibration before responses to NA, carbachol or field stimulation were tested. Following each dose, the tissue was washed until reproducible responses similar to the initial controls were obtained before testing the next highest dose of ketamine or cocaine. Responses in the presence of each dose of ketamine or cocaine were expressed as a percentage of the control preceding administration at that dose.

Comparison of responses before and after each dose of ketamine or cocaine *in vitro* or in the pithed rat was made using a paired *t*-test.

The following drugs were used: carbachol (Burroughs Wellcome); cocaine hydrochloride (Macarthys); ketamine hydrochloride (Ketalar, Parke Davis); noradrenaline bitartrate (Sigma) and pancuronium bromide (Pavulon, Organon).

## Results

### *Pithed rat arterial pressure responses*

Ketamine 2–50 mg/kg had a biphasic effect on the resting arterial pressure of the pithed rat, producing an

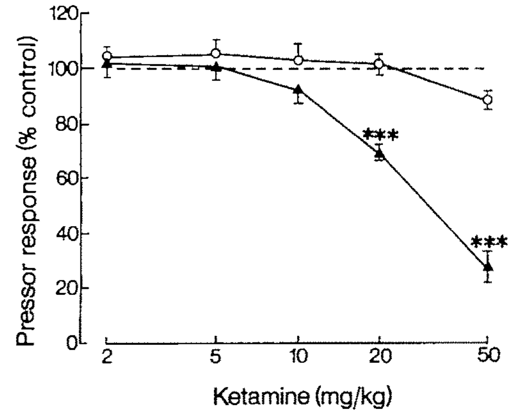


**Figure 1** Effect of ketamine on the arterial pressure response in the pithed rat to intravenous noradrenaline (○) or stimulation of the preganglionic sympathetic outflow at L1-2 (▲). NA dose 400 ng/kg; sympathetic stimulation, 10 mm electrode, supramaximal voltage, 1 ms pulses, 10 Hz for 10 s period. Responses 2 min after ketamine are expressed as a percentage of the control response preceding ketamine. Vertical lines show s.e. mean ($n=6$).

Statistical comparison using paired $t$ test of post-ketamine responses compared with controls: *** $P<0.001$.

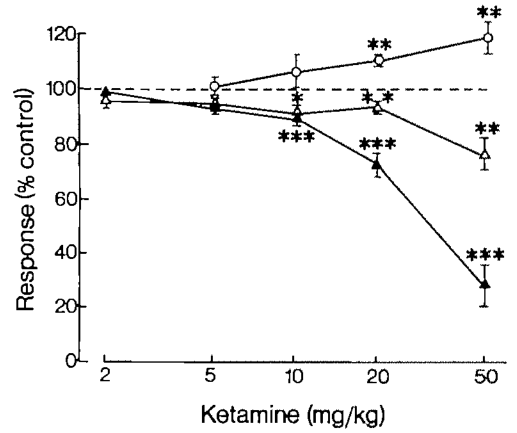


**Figure 2** Effect of ketamine on the anococcygeus muscle response in the pithed rat produced by intravenous noradrenaline (○), stimulation of the preganglionic sympathetic outflow at L1-2 (▲) or stimulation of postganglionic sympathetic fibres at S1-2 (△). NA dose 4 µg/kg; sympathetic stimulation, 10 mm electrode, supramaximal voltage, 1 ms pulses, 10 Hz for 10 s period. Responses 2 min after ketamine are expressed as a percentage of the control response preceding ketamine. Vertical lines show s.e. mean ($n=6$).

Statistical comparison using paired $t$ test of post-ketamine responses compared with controls: ** $0.01>P>0.001$; *** $P<0.001$.

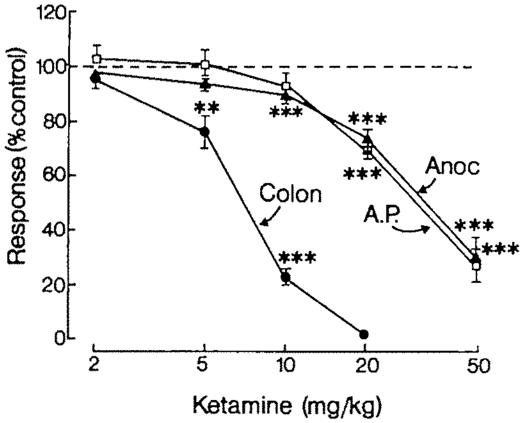


**Figure 3** Effect of ketamine on the responses in the pithed rat to stimulation of (1) the sympathetic outflow at L1–2; arterial pressure (A.P., □) and anococcygeus (Anoc, ▲); (2) the parasympathetic outflow at S1–2; colon (Colon, ●). Stimulation parameters: 10 mm electrodes, supramaximal voltage 1 ms pulses; in (1) 10 Hz, 10 s period, in (2) 10 Hz, 30 s period. Responses 2 min after ketamine are expressed as a percentage of the control response preceding ketamine. Vertical lines show s.e. mean ($n=6$).

Statistical comparison using a paired $t$ test of post-ketamine responses cf. controls: ** $0.01>P>0.001$; *** $P<0.001$.

initial transient fall in pressure followed by a short-lived rise. The initial fall was accompanied in every case by a fall in heart rate.

NA (400 ng/kg; i.v.) produced reproducible submaximal rises in arterial pressure of 65±3 mmHg, $n=20$. Ketamine 2–50 mg/kg had no significant effect on these responses (Figure 1).

Stimulation of the preganglionic sympathetic outflow (Ll-2) at 10 Hz for 10 s produced reproducible, submaximal rises in arterial pressure of 37±3 mmHg, $n=20$. Ketamine depressed these responses in a dose-dependent fashion which was statistically significant at 20 and 50 mg/kg (Figure 1).

### *Anococcygeus responses*

Ketamine 2–50 mg/kg had no effect on the resting tension of the anococcygeus muscle.

NA (4 µg/kg; i.v.) caused reproducible submaximal increases in anococcygeus tension of 2.9±0.2 g, $n=20$. Ketamine produced a dose-dependent potentiation of these responses which was statistically significant at 20 and 50 mg/kg (Figure 2).

Stimulation of the preganglionic sympathetic outflow (L1-2) at 10 Hz for 10 s produced reproducible, submaximal increases in anococcygeus tension of 6.6±0.2 g, $n=20$. Ketamine produced a

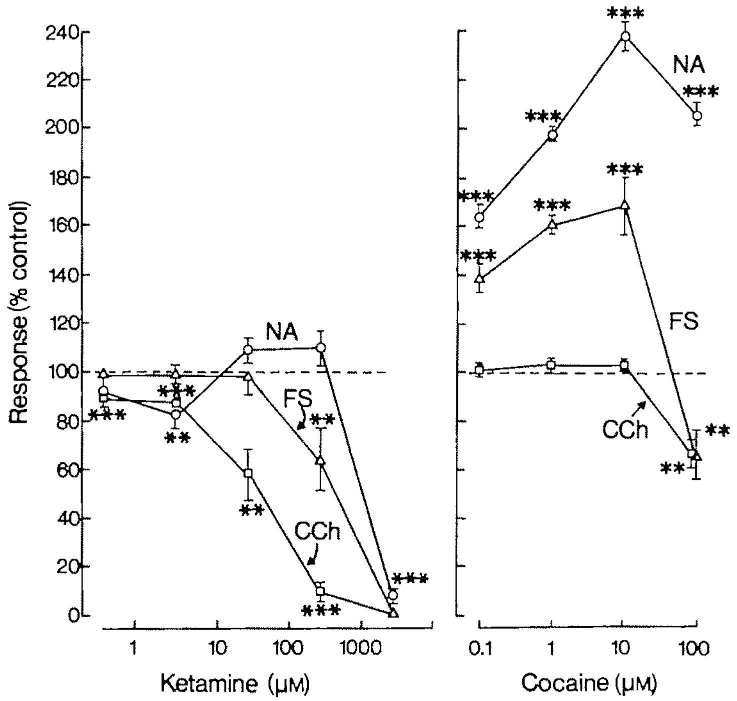


**Figure 4** Effects of ketamine and cocaine on the motor responses of the *in vitro* anococcygeus muscle, to noradrenaline (NA) 3 μM (○), carbachol (CCh) 1 μM (□) and field stimulation (FS, △). Responses in the presence of ketamine or cocaine are expressed as a percentage of control responses. Vertical lines show s.e. mean ($n = 6$).

Statistical comparison using a paired $t$ test of responses in the presence of ketamine cf. controls: ** $0.01 > P > 0.001$; *** $P < 0.001$.

dose-dependent inhibition of these responses which was statistically significant at 10 mg/kg and above (Figure 2). Stimulation with the pithing rod electrode at S1-2, (10 Hz, 10 s) which excites postganglionic sympathetic fibres to the anococcygeus (Gillespie & McGrath, 1973), produced reproducible submaximal responses in the anococcygeus of $6.4 \pm 0.3$ g, $n = 20$. Ketamine inhibited these responses in a dose-dependent manner which was statistically significant at 10 mg/kg and above. The extent of this inhibition was significantly less than in the case of preganglionic stimulation (Figure 2).

### *Colon responses*

Stimulation of the parasympathetic outflow at L5-6 produced reproducible increases in pressure in a balloon inside the colon. Ketamine inhibited these responses in a dose-dependent manner which was statistically significant at doses of 5 mg/kg and above. With a dose of 20 mg/kg of ketamine the response was completely extinguished in every experiment (Figure 3).

### *Anococcygeus* in vitro

*Effects of ketamine.* The dose-response curve in Figure 4 illustrates that ketamine (0.3 μM to 3 mM) inhibited responses to all three stimuli (NA, carbachol and field stimulation) in a dose-related manner but the magnitude of this inhibition was carbachol > field stimulation > NA. In addition there was a relative potentiation of the responses to NA in the range of concentration of ketamine 30 μM to 300 μM compared with the inhibition produced at lower or higher doses although this did not reach statistical significance compared with the pre-ketamine controls. In 7 out of 8 tissues tested, however, the response to NA was potentiated by either 30 μM or 300 μM ketamine.

*Effects of cocaine.* Cocaine significantly potentiated responses to NA and field stimulation with a maximum at 10 μM, whereas responses to carbachol were not significantly increased at any dose tested (Figure 4). The potentiation of NA responses increased to a maximum at cocaine 10 μM and declined with a further increase in cocaine concentration to 100 µM. The responses to carbachol were unaffected by cocaine at doses up to 10 µM, whereas they were depressed at 100 µM. Responses to field stimulation were potentiated by cocaine in a dose-related manner until the cocaine concentration reached 10 µM. At cocaine 100 µM, responses to field stimulation were significantly inhibited (Figure 4).

## Discussion

These results lend little support to the hypothesis that ketamine might raise blood pressure by peripheral potentiation of sympathetic nerve responses. The only evidence found in this study for a potentiation of adrenergic responses *in vivo* by ketamine was a small increase in the response of the anococcygeus muscle to NA with the highest doses of ketamine of 20 or 50 mg/kg (i.v.). However, the response of the vasculature to NA showed no potentiation at any dose and the responses of both the vasculature and the anococcygeus muscle to stimulation of the preganglionic sympathetic outflow were depressed by ketamine in a dose-dependent manner. *In vitro*, the response to NA of the anococcygeus muscle was depressed in a dose-dependent manner although in the range of ketamine concentration 30 µM to 300 µM, the responses of some preparations were increased, perhaps indicating a small potentiating effect of ketamine in this range. The response to field stimulation of the adrenergic motor nerves in the anococcygeus was, however, also depressed by ketamine in a dose-dependent manner.

This does not conflict with the evidence that ketamine can block neuronal NA uptake *in vitro* (Nedergaard, 1973; Montel *et al.*, 1973). However, at the high doses of ketamine required to produce potentiation of NA responses, widespread depressant effects contribute to a net effect in the whole animal which would act against any pressor effect. Thus the responses to stimulation of post-ganglionic sympathetic nerves to the anococcygeus are depressed *in vivo* or *in vitro* and responses to preganglionic sympathetic nerve stimulation to the anococcygeus or the vascular system are depressed to an even greater degree. This suggests depressant effects at two sites at least in the sympathetic pathway *viz.* the ganglion synapse and the postganglionic terminal synapse. When the parasympathetic pathway to the colon is considered, the degree of depression by ketamine was greater still and occurred at doses of ketamine as low as 5 mg/kg (i.v.) (Figure 3).

The effects of cocaine on the *in vitro* anococcygeus illustrate that with the protocol employed it is possible to detect potentiation of adrenergic responses due to blockade of neuronal uptake of NA. It has previously been demonstrated that cocaine 1 µM potentiates NA responses in this tissue (Gibson & Pollock, 1973). The present study confirms this and illustrates that this effect increases with increasing doses of cocaine up to 10 µM as also does the potentiation of the effects of adrenergic nerve stimulation. At 100 µM cocaine, however, the local anaesthetic action predominates and both nerve-mediated and post-synaptic responses are depressed. *In vivo*, Muscholl (1961) has shown that cocaine (10–20 mg/kg) can potentiate the pressor response to NA in the pithed rat and McGrath (1973) has shown that cocaine (1 mg/kg) can potentiate the vasopressor and anococcygeus muscle responses to stimulation of the lumbar sympathetic outflow of the pithed rat. Simpson (1975) has shown also that the $ID_{50}$ for blockade of neuronal NA uptake into the heart of the pithed rat is 3 mg/kg cocaine.

The relative potency of ketamine and cocaine at blocking neuronal NA uptake is relevant to any possible mechanism contributing to a pressor response in the whole animal. From the present study and from the earlier work on blockade of neuronal NA uptake (Nedergaard, 1973; Montel *et al.*, 1973) ketamine appears to be 30 to 100 times less potent than cocaine on a molar basis at potentiating NA responses. If, therefore, 1–20 mg/kg (3.3–66 µmol/kg) cocaine is required to produce potentiation of responses *in vivo*, it is likely that the dose of ketamine to produce the same effect would be at least in the order of 20–100 mg/kg (89–440 µmol/kg). This is confirmed by the results for the anococcygeus in the pithed rat where only doses of ketamine of 20 and 50 mg/kg (i.v.) produced potentiation of NA responses and any higher doses stopped the heart by a direct depression of the myocardium (Dowdy & Kaya, 1968). In contrast, the anaesthetic doses of ketamine which raise arterial pressure in man and several animal species are of the order of 2–5 mg/kg (i.v.) (McCarthy *et al.*, 1965; Domino *et al.*, 1965; Traber & Wilson, 1969; McGrath, Mackenzie & Millar, 1975a). In addition, since ketamine does not block the baroreceptor reflex controlling sympathetic tone (McGrath, Mackenzie & Millar, 1975b), any small effect due to a peripheral action would not be maintained due to the 'buffering' effect of this reflex.

We suggest that ketamine, like cocaine, possesses the dual properties of neuronal NA uptake blockade and local anaesthetic-type depression of synaptic transmission, but that whereas cocaine possesses the former property at low doses and the latter at high doses, with ketamine the optimal doses for each property are close together and, therefore, the net effect will depend on the system under investigation.

In conclusion, the results suggest that ketamine does not produce pressor effects in anaesthetic doses by a potentiation of adrenergic responses. Doses which do produce some potentiation of peripheral adrenergic responses are higher than anaesthetic doses and produce predominantly depressant effects on the peripheral autonomic nervous system.

We are grateful to the Wellcome Trust for a Fellowship to J.C. McGrath and a scholarship to A.S. Clanachan, to Professor J.S. Gillespie and Professor R.A. Millar for their advice and support and the Rankin Fund of the University of Glasgow for equipment.

## References

CHANG, P., CHAN, K.E. & GANENDRAN, A. (1969). Cardiovascular effects of 2-(*o*-chlorophenyl)-2-methylaminocyclohexanone (CI-581) in rats. *Br. J. Anaesth.*, **41**, 391–395.

CLANACHAN, A.S. & McGRATH, J.C. (1975). Effects of ketamine on the peripheral sympathetic nervous system of the rat. *Br. J. Pharmac.*, **55**, 245P.

DOMINO, E.F., CHODOFF, P. & CORSSEN, G. (1965). Pharmacologic effects of CI-581, a new dissociative anaesthetic in man. *Clin. Pharmac. Ther.*, **6**, 279–291.

DOWDY, ELIZABETH G. & KAYA, K. (1968). Studies of the mechanism of cardiovascular responses to CI-581. *Anaesthesiology*, **29**, 931–943.

GIBSON, A. & POLLOCK, D. (1973). The effects of drugs on the sensitivity of the rat anococcygeus muscle to agonists. *Br. J. Pharmac.*, **49**, 506–513.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmac.*, **45**, 404–416.

GILLESPIE, J.S. & McGRATH, J.C. (1973). The spinal origin of the motor and inhibitory innervation of the rat anococcygeus muscles. *J. Physiol., Lond.*, **230**, 659–672.

GILLESPIE, J.S., MacLAREN, A. & POLLOCK, D. (1970). A method of stimulating different segments of the autonomic outflow from the spinal column to various organs in the pithed cat and rat. *Br. J. Pharmac.*, **40**, 257–267.

IVERSEN, L.L. (1973). Catecholamine uptake processes. *Br. med. Bull.*, **29**, 130–135.

McCARTHY, D.A., CHEN, G., KAUMP, D.H. & ENSOR, C. (1965). General anaesthetic and other pharmacological properties of 2-(*o*-chlorophenyl)-2-methylaminocyclohexanone HCl (CI-581). *J. New Drugs*, **5**, 21–33.

McGRATH, J.C. (1973). The inhibitory and motor innervation of the anococcygeus muscle. Ph.D. Thesis. University of Glasgow.

McGRATH, J.C., MacKENZIE, J.E. & MILLAR, R.A. (1975a). Circulatory response to ketamine: Dependence on respiratory pattern and background anaesthesia in the rabbit. *Br. J. Anaesth.*, **47**, 1149–1156.

McGRATH, J.C., MacKENZIE, J.E. & MILLAR, R.A. (1975b). Effects of ketamine on central sympathetic discharge and the baroreceptor reflex during mechanical ventilation. *Br. J. Anaesth.*, **47**, 1141–1147.

MONTEL, H., STARKE, K., GORLITZ, B-D., & SCHUMANN, H.J. (1973). Tierexperimentelle Untersuchungen zur Wirkung des Ketamins auf periphere sympathische Nerven. *Anaesthesist*, **22**, 111–116.

MUSCHOLL, E. (1961). Effect of cocaine and related drugs on the uptake of noradrenaline by heart and spleen. *Br. J. Pharmac. Chemother.*, **16**, 352–359.

NEDERGAARD, O.A. (1973). Cocaine-like effect of ketamine on vascular adrenergic neurones. *Eur. J. Pharmac.*, **23**, 153–161.

SIMPSON, L.L. (1975). Blood pressure and heart rate responses evoked by d and l-amphetamine in the pithed rat preparation. *J. Pharmac. exp. Ther.*, **193**, 149–159.

TRABER, D.L. & WILSON, R.D. (1969). Involvement of the sympathetic nervous system in the pressor response to ketamine. *Anaesth. Analg.*, **48**, 248–252.

*(Received March 23, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 253–259

# RENAL FUNCTION AND RENAL VENOUS PROSTAGLANDIN CONCENTRATIONS DURING DIFFERENT STAGES OF EXPERIMENTAL RENAL HYPERTENSION IN THE RAT

G.W. SMITH & LILIANA I. SOMOVA[1]
Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ

1 Renal hypertension was produced in rats and the changes in renal function, renal venous prostaglandin $E_2$ and $F_{2\alpha}$ concentrations and secretion rates were studied at various times.

2 Renal plasma flow transiently fell in the ischaemic kidney 2 weeks after clamping, whilst that of the other kidney did not change. Glomerular filtration rate remained constant in both kidneys throughout the entire study.

3 Prostaglandins $E_2$ and $F_{2\alpha}$ concentrations rose in the venous plasma from the ischaemic kidney, but did not change in the other kidney and appeared to be inversely related to renal plasma flow.

4 Calculated secretion rate of both prostaglandins fell in the ischaemic kidney and did not change in the other kidney.

5 Clamping the second kidney, two weeks after the first, caused a further elevation in blood pressure, a fall in renal plasma flow and a fall in prostaglandin secretion rate in both kidneys.

6 The implications of these prostaglandin changes are discussed.

## Introduction

Acute renal ischaemia increases the concentration of prostaglandin E-like material in renal venous blood of the dog and this has been interpreted as a rise in output of renal prostaglandins (McGiff, Crowshaw, Terragno, Lonigro, Strand, Williamson, Lee & Ng, 1970). Such a mechanism may protect the kidney from the antinatriuretic/vasoconstrictor influence of the renin and sympathetic systems (Lonigro, Terragno, Malik & McGiff, 1973).

The contralateral renal prostaglandins have also been assigned a role. The contralateral kidney of the Goldblatt hypertensive dog has an antihypertensive function, independent of excretion (Grollman, Muirhead & Vanatta, 1949). Such a system is also present in the rat kidney, when perfused at hypertensive pressures (Tobian, Schonning & Seefeldt, 1964). Lee (1973) believes that the renal prostaglandins may play a role.

No study has yet examined renal venous prostaglandin concentrations in chronic renal ischaemia of experimental origin nor attempted to evaluate the rate of output of renal prostaglandins.

In this study we examined renal function and renal venous prostaglandin $E_2$ and $F_{2\alpha}$ concentrations of both kidneys of rats at various stages after clamping the left renal artery and also after clamping both renal arteries.

## Methods

One hundred and thirty six male and female Wistar rats, of approximately similar size and fed on a standard laboratory diet, with water *ad lib.*, were divided into seven experimental groups, 30 rats serving as controls. Renal hypertension was induced by clipping the left renal artery with a small silver clip (first operation) and in some rats, clipping the right renal artery (second operation) two weeks later. The rats were killed at various stages after clipping as follows: group 1, 20 rats killed 1 week after the first operation; group 2, 20 rats killed 2 weeks after the first operation; group 3, 20 rats killed 3 weeks after the first operation; group 4, 20 rats killed 4 weeks after the first operation; group 5, 20 rats killed 1 week after the second operation; group 6, 20 rats killed 2 weeks after the second operation and group 7, 16 rats killed 10 weeks after the second operation (the chronic hypertensive group).

---

[1] Present address: Research Group of Hypertension, Medical Academy, Sofia 31, Bulgaria.

The rats were weighed weekly and arterial blood pressure was measured by the indirect tail-cuff method using a Rat Blood Pressure Monitor (Huntingdon Instruments, England).

From each group, 6 to 8 rats were used for the estimation of bilateral renal function, namely renal plasma flow (RPF) and glomerular filtration rate (GFR) and the remaining rats were used for the estimation of renal venous prostaglandin concentrations.

RPF and GFR were estimated by means of the clearances of para-aminohippurate (PAH) and of inulin respectively. The rats were anaesthetized with pentobarbitone (40 mg/kg i.p.) and tracheotomized. The left subclavian vein was catheterized for the infusion of the clearance solution at a rate of 0.05 ml/min by means of a motor driven syringe (Braun). The clearance solution was composed of 1% inulin, 1% PAH and 2% $Na_2SO_4$ dissolved in 0.9% w/v NaCl solution and buffered to pH 7.4 with bicarbonate.

Both ureters were catheterized after the method of Mercer (1971) and a 10 min urine sample was collected from each ureter 50 min after the infusion had begun. After heparinization (1000 u/kg), blood samples (0.5 ml) were removed from a carotid artery catheter 50 and 60 min after the start of the infusion, for the calculation of mean plasma PAH and inulin concentrations, which we tried to hold at about 7 and 30 mg% respectively.

The blood was centrifuged in Eppendorf tubes and 0.2 ml of plasma removed and subjected to plasma protein precipitation with Somogyi reagent. The urine was carefully transferred to a volumetric flask and diluted to 100 ml. Diluted urine and protein-free plasma were analysed for inulin and PAH using a resorcinol method for the former and *N* (1-napthyl)-ethylene diamine as a coupling reagent for the latter and both were compared with standard solutions in a spectrophotometer (Bausch and Lomb, SP 20, U.S.A.).

The clearances of PAH and inulin were calculated and expressed in terms of body weight. The extraction ratio for PAH was not taken into account. Girndt & Ochwadt (1969) have shown that 4 weeks after clamping a renal artery, PAH extraction was unaltered in both the clamped and the contralateral kidneys (0.84 approx.).

Blood samples for the estimation of prostaglandins were drawn from the renal veins of anaesthetized rats after a midline laparotomy and catheterization of the inferior vena cava. Within each group, the left and right renal venous blood samples were pooled separately (final volume about 10 ml). A known amount of deuterium isotope, 1 µg $d_4$ prostaglandin $E_2$ and 500 ng $d_4$ prostaglandin $F_{2\alpha}$ was added to each plasma sample and the prostaglandins were extracted and subjected to silicic acid column chromatography (Poyser, 1972). Methyl ester/methoxime/trimethylsilyl and methyl ester/trimethylsilyl derivatives were made of the extracted prostaglandins $E_2$ and $F_{2\alpha}$ respectively (Thompson, Los & Horton, 1970; Blatchley, Donovan, Horton & Poyser, 1972). Using multiple ion detection (Finnigan 3000D mass

**Table 1** Changes in blood pressure (mmHg) and prostaglandin (PG) level (ng/ml) in renal venous blood of control and renal hypertensive rats

| Group | (n) | Blood pressure<br>Systolic | <br>Diastolic | $PGE_2$<br>Left renal vein | <br>Right renal vein | $PGF_{2\alpha}$<br>Left renal vein | <br>Right renal vein |
|---|---|---|---|---|---|---|---|
| Controls | (30) | 126±2.73 | 100±2.55 | 4.0 | 3.8 | 2.7 | 2.2 |
| 1 | (20) | 139±5.17<br>$P<0.05$ | 111±4.69<br>$P<0.05$ | 4.4 | 2.7 | 3.7 | 2.1 |
| 2 | (20) | 142±4.71<br>$P<0.01$ | 112±3.91<br>$P<0.02$ | 5.6 | 3.6 | 4.3 | 2.7 |
| 3 | (20) | 148±5.13<br>$P<0.001$ | 123±4.26<br>$P<0.001$ | 4.6 | 2.6 | 3.2 | 2.0 |
| 4 | (20) | 148±5.62<br>$P<0.001$ | 118±4.14<br>$P<0.001$ | 3.2 | 5.0 | 1.2 | 2.9 |
| 5 | (20) | 152±4.57<br>$P<0.001$ | 121±3.87<br>$P<0.001$ | 3.0 | 4.5 | 1.6 | 4.4 |
| 6 | (20) | 193±7.61<br>$P<0.001$ | 153±7.84<br>$P<0.001$ | 4.7 | 6.2 | 2.7 | 3.1 |
| 7 | (16) | 183±5.55<br>$P<0.001$ | 150±5.14<br>$P<0.001$ | 2.9 | 4.5 | 1.9 | 3.3 |

*n* is the number of rats in each group. Values are mean ± s.e. Groups 1 to 4 had clipped left kidneys. Groups 5 to 7 had both kidneys clipped.

spectrometer), the ratio of proton to deuterium peaks of the prostaglandin derivatives was obtained and compared with a standard calibration curve over the range of 1 to 1000 ng of protium. (Hensby & Naylor, 1974.)

Statistical analysis of the results was performed by Student's two-tailed *t* test, except for the renal function results, where a one-tailed *t* test was used (Dixon & Massay, 1969). Values are expressed as mean ± s.e. and only probabilities less than 0.05 were accepted as being statistically significant.

## Results

### *Development of hypertension*

One week after the production of renal ischaemia (groups 1 to 4), there was a significant rise in blood pressure, which reached a peak by the third week (group 3). Systolic blood pressure rose from 126 ± 2.73 mmHg to 148 ± 5.13 mmHg ($P < 0.001$) and diastolic pressure increased from 100 ± 2.55 mmHg to 123 ± 4.26 mmHg ($P < 0.001$) three weeks after clamping (group 3) (Table 1).

Cardiac hypertrophy also occurred over this period as seen by the rise in the heart/body weight ratio (Table 2). The left kidney/body weight ratio rose in group 1 rats but fell in group 2 and was not significantly different from control in later groups. The right kidney hypertrophied in each of the unilaterally clamped groups, except for group 2 (Table 2).

An inverse relationship between renal mass and the level of systemic hypertension was noted in almost all cases.

During ischaemia of the right kidney, as well as of the left, (groups 5, 6 and 7), systolic and diastolic pressures rose further, within the range 126/100 to 183/150 mmHg 10 weeks after the second operation (the chronic hypertensive group).

The left, earlier ischaemic kidney, was now firm and shrunken, especially in the chronic group (group 7). The right kidney, which had previously been hypertrophied before the second operation, was now not significantly different from control. Cardiac hypertrophy was very marked in these bilaterally ischaemic rats.

### *Renal function changes*

After unilateral renal ischaemia, RPF of the clamped kidney fell significantly 2 weeks after clamping (group 2), from 8.23 ± 1.28 ml $\text{min}^{-1}$ $\text{kg}^{-1}$ to 4.28 ± 1.55 ml $\text{min}^{-1}$ $\text{kg}^{-1}$ ($P < 0.05$) and subsequently rose again to control values (Table 3). The contralateral kidney showed no significant change in RPF at this time. Due to the well maintained GFR, the filtration fraction (FF) rose significantly only in the ischaemic kidneys of group 2 rats.

On clamping the right kidney, RPF fell 2 weeks later in group 6 from 9.12 ± 1.31 ml $\text{min}^{-1}$ $\text{kg}^{-1}$ to 3.43 ± 0.25 ml $\text{min}^{-1}$ $\text{kg}^{-1}$ ($P < 0.025$) (Table 3). Left RPF also fell, from 8.23 ± 1.29 ml $\text{min}^{-1}$ $\text{kg}^{-1}$ to 4.44 ± 0.44 ml $\text{min}^{-1}$ $\text{kg}^{-1}$ ($P < 0.025$). This fall was also significant ($P < 0.0005$) when compared with group 4, which had undergone exactly the same procedures and time course, except for the second

**Table 2** Average organ/body weights in control and experimental rats.

| *Group* | (n) | *Body weight (g)* | *Heart/body weight* | *Left kidney/ body weight* | *Right kidney/ body weight* |
|---|---|---|---|---|---|
| Controls | (30) | 257 ± 12 | 32.4 ± 0.6 | 35.2 ± 0.5 | 35.9 ± 0.6 |
| 1 | (20) | 226 ± 8 | 36.7 ± 1.2<br>$P < 0.01$ | 39.8 ± 1.8<br>$P < 0.02$ | 42.7 ± 2.0<br>$P < 0.001$ |
| 2 | (20) | 228 ± 15 | 35.4 ± 0.7<br>$P < 0.01$ | 32.6 ± 1.2<br>$P < 0.05$ | 35.5 ± 1.4<br>$P > 0.5$ |
| 3 | (20) | 248 ± 8 | 34.2 ± 0.5<br>$P < 0.05$ | 35.3 ± 1.8<br>$P > 0.5$ | 40.4 ± 1.2<br>$P < 0.01$ |
| 4 | (20) | 212 ± 11 | 38.5 ± 1.2<br>$P < 0.001$ | 34.8 ± 1.3<br>$P > 0.5$ | 41.3 ± 1.3<br>$P < 0.001$ |
| 5 | (20) | 292 ± 15 | 33.4 ± 1.4<br>$P > 0.5$ | 31.3 ± 1.1<br>$P < 0.01$ | 35.5 ± 1.2<br>$P > 0.5$ |
| 6 | (20) | 188 ± 13 | 41.5 ± 1.4<br>$P < 0.001$ | 31.6 ± 1.9<br>$P < 0.05$ | 40.1 ± 2.3<br>$P > 0.05$ |
| 7 | (16) | 187 ± 16 | 41.4 ± 2.1<br>$P < 0.001$ | 30.2 ± 2.1<br>$P < 0.02$ | 39.8 ± 2.7<br>$P > 0.1$ |

Body weights are expressed in grams. Organ/body weights are expressed $\times 10^{-4}$. *n* is the number of rats in each group. Values are mean ± s.e. Groups 1 to 4 had clipped left kidneys. Groups 5 to 7 had both kidneys clipped.

operation. As GFR was so well maintained, FF rose in the left kidneys of group 6. In group 7, the chronic hypertensive group, no difference was detected between either kidney and control.

### *Prostaglandins $E_2$ and $F_{2\alpha}$ renal venous concentrations*

After the first operation, left renal venous prostaglandin $E_2$ and $F_{2\alpha}$ concentrations were higher than control, whereas the concentrations in the right renal venous plasma were similar or slightly lower than control, except for group 4 (see Table 1, groups 1 to 4).

After the second operation, prostaglandins $E_2$ and $F_{2\alpha}$ concentrations rose in the right renal venous plasma 1 week later (group 5). By the second week (group 6), prostaglandin $E_2$ had risen even more in the right renal venous plasma but prostaglandin $F_{2\alpha}$ had fallen, although still above control. Both prostaglandins rose transiently in the left renal venous plasma. In the chronic hypertensive rats (group 7), right renal venous prostaglandin $E_2$ and $F_{2\alpha}$ concentrations were higher than control, in contrast to the left renal venous concentrations, which were lower than control.

### *Prostaglandin secretion rates*

Secretion rate of each prostaglandin may be calculated from the product of the total mean RPF and the prostaglandin concentration for each group since prostaglandins appear to be released on synthesis (Änggård, Bohman, Griffin, Larsson & Maunsbach, 1972). This assumes that all the arterial prostaglandins presented to the kidney are cleared in one passage because of the high activity of the metabolizing enzyme, 15 hydroxy prostaglandin dehydrogenase, in the renal cortex (Änggård, Larsson & Samuelsson, 1971). The clamped kidney showed a fall in secretion rate of prostaglandin $E_2$ from 8.68 ng/min to 6.24, 5.57, 8.51 and 4.84 ng/min in groups 1, 2, 3 and 4 respectively. Prostaglandin $F_{2\alpha}$ secretion rate also fell, from 5.86 ng/min to 5.25, 4.28, 5.92 and 1.81 ng/min in each of the above respective groups (see Table 4). The contralateral kidney of these groups showed no change in secretion rate of either prostaglandin. After ischaemia of the right kidney, secretion rate of both prostaglandins began to fall in the right kidney and continued falling in the left kidney (groups 5, and 6). A subnormal secretion rate of both prostaglandins existed in the two kidneys of the chronic hypertensive group (group 7).

## **Discussion**

Four weeks after clamping a renal artery, the resultant hypertension was associated with normal PAH and inulin clearances in both the clamped and contralateral kidneys of rats (Girndt & Ochwadt, 1969). However, medullary blood flow was elevated in the contralateral kidney as determined by the $^{86}$Rubidium method. Our results are essentially in agreement with these findings. However, at variance with this, Kramer & Ochwadt (1974) reported that rat kidneys clamped 6 to 10 weeks previously, had lower PAH and inulin clearances in comparison with the contralateral kidney.

**Table 3** Glomerular filtration rate and renal plasma flow

| | | *Glomerular filtration rate* | | *Renal plasma flow* | | *Filtration fraction* | |
|---|---|---|---|---|---|---|---|
| *Group* | (n) | *Left kidney* | *Right kidney* | *Left kidney* | *Right kidney* | *Left kidney* | *Right kidney* |
| Controls | (10) | 1.80 ± 0.25 | 2.20 ± 0.28 | 8.23 ± 1.29 | 9.12 ± 1.31 | 0.24 ± 0.03 | 0.27 ± 0.05 |
| 1 | (8) | 1.50 ± 0.26<br>$P > 0.2$ | 2.66 ± 0.28<br>$P > 0.05$ | 6.34 ± 1.32<br>$P > 0.05$ | 10.14 ± 1.25<br>$P > 0.2$ | 0.28 ± 0.03<br>$P > 0.2$ | 0.30 ± 0.04<br>$P > 0.2$ |
| 2 | (8) | 1.57 ± 0.39<br>$P > 0.2$ | 2.41 ± 0.59<br>$P > 0.2$ | 4.28 ± 1.55<br>$P < 0.05$ | 8.56 ± 2.92<br>$P > 0.2$ | 0.41 ± 0.07<br>$P > 0.05$ | 0.38 ± 0.05<br>$P > 0.1$ |
| 3 | (8) | — | — | 7.46 ± 1.12<br>$P > 0.2$ | 9.00 ± 1.39<br>$P > 0.2$ | — | — |
| 4 | (8) | 1.88 ± 0.18<br>$P > 0.02$ | 2.09 ± 0.33<br>$P > 0.2$ | 7.59 ± 0.35<br>$P > 0.2$ | 10.95 ± 2.25<br>$P > 0.2$ | 0.21 ± 0.02<br>$P > 0.5$ | 0.28 ± 0.06<br>$P > 0.5$ |
| 5 | (8) | 1.62 ± 0.15<br>$P > 0.2$ | 2.09 ± 0.23<br>$P > 0.2$ | 5.85 ± 0.95<br>$P > 0.05$ | 7.14 ± 0.89<br>$P > 0.05$ | 0.30 ± 0.02<br>$P > 0.05$ | 0.31 ± 0.01<br>$P > 0.2$ |
| 6 | (7) | 1.84 ± 0.27<br>$P > 0.2$ | 1.45 ± 0.35<br>$P > 0.05$ | 4.44 ± 0.44<br>$P < 0.025$ | 3.43 ± 0.25<br>$P < 0.025$ | 0.42 ± 0.09<br>$P \geqslant 0.05$ | 0.45 ± 0.14<br>$P > 0.2$ |
| 7 | (6) | 1.50 ± 0.16<br>$P > 0.2$ | 1.68 ± 0.19<br>$P > 0.05$ | 5.49 ± 0.79<br>$P \ngtr 0.05$ | 6.30 ± 0.75<br>$P > 0.05$ | 0.34 ± 0.06<br>$P > 0.1$ | 0.37 ± 0.08<br>$P > 0.1$ |

Values are mean ± s.e. Clearances are expressed in ml min$^{-1}$ kg$^{-1}$ body weight. *n* is the number of rats in each group. Groups 1 to 4 had clipped left kidneys. Groups 5 to 7 had both kidneys clipped.

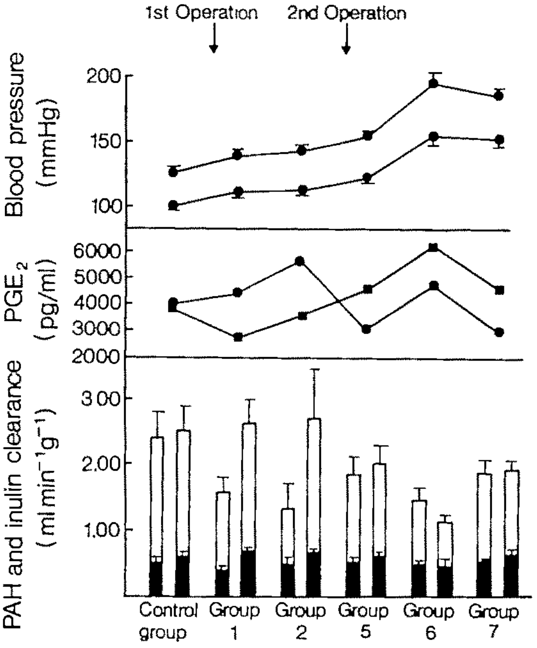


**Figure 1** Systolic and diastolic blood pressures (mmHg), renal venous prostaglandin $E_2$ concentrations of the left (●) and right (■) kidneys and RPF (white bars) and GFR (black bars) of the left and right kidneys respectively. In each pair, at different stages of hypertension. Values are mean ± s.e.

McGiff *et al.* (1970) propose that the kidney releases prostaglandin-like material in response to reduced renal blood flow caused by constricting the renal artery. Herbaczynska-Cedro & Vane (1973) obtained a rise in renal venous prostaglandin E-like material on reducing perfusion pressure and renal blood flow, in the pump perfused canine kidney and suggested that increased secretion of prostaglandin E mediated autoregulation of blood flow since indomethacin abolished autoregulation and the prostaglandin release. However, this concept may be no longer tenable in the intact, naturally perfused kidney (Owen, Ehrhart, Weidner, Haddy & Scott, 1974; Venuto, O'Dorisio, Ferris & Stein, 1975; Anderson, Taher, Cronin, McDonald & Schrier, 1975). Using essentially the same approach as Herbaczynska-Cedro & Vane, autoregulation was found to be resistant to meclofenamate (10 mg/kg, i.v.) although absolute flow fell (Dighe, Hall, Smith & Ungar, unpublished results). It remains to be seen whether rat kidney behaves in a similar manner.

Renal prostaglandins may have a natriuretic role and protect the kidney against the vasoconstrictor/antidiuretic influence of the renin and sympathetic systems (McGiff & Nasjletti, 1973; Lonigro, *et al.*, 1973). However, in the isolated kidney of the rat, unlike other species, prostaglandin $E_2$ potentiates renal sympathetic nerve stimulation and at higher doses causes vasoconstriction similar to that induced by prostaglandin $F_{2\alpha}$ (Malik & McGiff, 1975).

We have shown that prostaglandin $E_2$ and $F_{2\alpha}$ concentrations rise in the plasma from ischaemic kidneys, whereas the contralateral renal venous concentrations remain unaltered or may perhaps fall slightly. As Figure 1 shows, there is an inverse relationship between prostaglandin $E_2$ concentration and RPF for both kidneys. This may either be a result or a cause of the blood flow changes. Thus an increase in prostaglandin $E_2$ or $F_{2\alpha}$ concentrations may reduce RPF by the mechanism described by Malik & McGiff (1975) and conversely a fall in RPF may be due to a fall in prostaglandin concentration.

Alternatively, the concentration changes may be a consequence of flow changes. This is supported by the findings of Beckman & Zehr (1975) in the dog. On halving renal blood flow, renal venous prostaglandin

**Table 4** Estimated prostaglandin (PG) secretion rates (ng/min) in kidneys from control and experimental rats

| | $PGE_2$ | | $PGF_{2\alpha}$ | |
|---|---|---|---|---|
| *Group* | *Left kidney* | *Right kidney* | *Left kidney* | *Right kidney* |
| Control | 8.68 | 8.71 | 5.86 | 5.04 |
| 1 | 6.24 | 6.82 | 5.25 | 5.31 |
| 2 | 5.57* | 8.06 | 4.28* | 6.05 |
| 3 | 8.51 | 6.65 | 5.93 | 5.11 |
| 4 | 4.84 | 11.41 | 1.81 | 6.62 |
| 5 | 5.62 | 9.25 | 2.64 | 9.04 |
| 6 | 4.13* | 5.51* | 2.37* | 2.75* |
| 7 | 3.08 | 6.67 | 2.02 | 4.81 |

Values were obtained from the product of total mean RPF of each group and the respective prostaglandin concentration and are expressed in nanograms per minute. Groups 1 to 4 had left kidneys clipped. Groups 5 to 7 had both kidneys clipped.

* Signifies secretion rates where a statistically significant fall in RPF was observed.

$E_2$, as detected by radioimmunoassay, rose, but calculated secretion rate fell. We have also obtained similar results in the pump perfused canine kidney (Dighe, Hall, Smith and Ungar, unpublished observations). This is in contrast to what is generally believed and demonstrates the possible fallacy of equating concentration changes with secretion changes especially when concentration and flow change in an inverse manner as they do in the clamped kidney.

Our results also suggest that secretion rates of prostaglandin $E_2$ and $F_{2\alpha}$ fall in the chronically ischaemic rat kidney, with little change in the contralateral kidney. It must be borne in mind that our secretion figures were obtained from two groups of rats, namely those used for prostaglandin estimations and those for renal function tests, but there is no reason why these rats should not have been representative.

A reduced release of prostaglandin E-like material by the isolated kidney of one kidney Goldblatt rats, perfused at constant flow and challenged with pressor doses of noradrenaline, has been reported (Leary, Ledingham & Vane, 1974). Reduced synthetic capacity of prostaglandins was also found in the kidneys of renal and spontaneously hypertensive rats (Sirvios & Gagnon, 1974).

In contrast, using radioimmunoassay, Jaffe, Parker, Marshall & Needleman (1972) observed a rise in prostaglandin E in both the clamped and contralateral kidneys of chronic hypertensive rats. Arterial levels were also raised, suggesting a genuine increase in synthesis, but not necessarily from the kidney.

In earlier investigations (Somova, 1971; 1973) an increase in prostaglandin E-like material was seen in the ischaemic kidney of the rat during the acute phase of hypertension, with a decrease in the chronic stages.

The contralateral kidney has an endocrine antihypertensive role, distinct from excretion as demonstrated by Grollman *et al.* (1949) and Kolff (1958) in the dog. Similar findings have been reported in the rat kidney, when introduced into the circulation of the hypertensive rat (Tobian *et al.*, 1964). Muirhead, Germain, Brooks & Stephenson (1973) have attributed this property to the renal medulla and probably to the renal interstitial cells, which have been shown to synthesize prostaglandins. Rabbit renomedullary lipid is antihypertensive in rat (Muirhead, Leach, Daniels & Hinman, 1968) and rat renal medulla, especially that from hypertensive rats, is antihypertensive when implanted into hypertensive rats (Tobian & Azar, 1971). However, the antihypertensive principle may not be the renal prostaglandins but the neutral lipid (ANRL), which the renal interstitial cells also elaborate (Muirhead, Leach, Germain, Byers & Armstrong, 1974).

Certainly our results do not agree with the concept that the antihypertensive function of the kidney is due to an enhanced release of prostaglandin $E_2$ since secretion rate in the contralateral kidney does not change.

The authors are indebted to Dr C.N. Hensby for performing the prostaglandin estimations. L.I.S. was generously supported by a British Council Grant. G.W.S. holds an M.R.C. scholarship.

## References

ANDERSON, R.J., TAHER, M.S., CRONIN, R.E., McDONALD, K.M. & SCHRIER, R.W. (1975). Effect of $\beta$-adrenergic blockade and inhibitors of angiotensin II and prostaglandins on renal autoregulation. *Am. J. Physiol.*, **229**, 731–736.

ÅNGGÅRD, E., BOHMAN, S.O., GRIFFIN, J.E. III, LARSSON, C. & MAUNSBACH, A.B. (1972). Subcellular localization of the prostaglandin system in the rabbit renal papilla. *Acta physiol. scand.*, **84**, 231–246.

ÅNGGÅRD, E., LARSSON, C. & SAMUELSSON, B. (1971). The distribution of 15-hydroxy prostaglandin dehydrogenase and prostaglandin-$\Delta^{13}$-reductase in tissues of the swine. *Acta physiol. scand.*, **81**, 396–404.

BECKMAN, M.L. & ZEHR, J.E. (1975). Partition of PGE between renal venous plasma and urine during renal ischaemia. *Prostaglandins*, **9**, 721–736.

BLATCHLEY, F.R., DONOVAN, B.T., HORTON, E.W. & POYSER, N.L. (1972). The release of prostaglandins and progestin into the utero-ovarian venous blood of guinea-pigs during the oestrous cycle and following oestrogen treatment. *J. Physiol. Lond.*, **223**, 69–88.

DIXON, W.J. & MASSAY, F.J. (1969). *Introduction to statistical analysis*. Third edition. p. 116. New York: McGraw-Hill Book Company.

GIRNDT, J. & OCHWADT, B. (1969). Durchblutung des Nierenmarkes, Gesamtnierendurchblutung und corticomedulläre Gradienten beim experimentallen renalen Hochdruck der Ratte. *Pflügers Archiv.*, **313**, 30–42.

GROLLMAN, A., MUIRHEAD, E.E. & VANATTA, J. (1949). Role of the kidney in the pathogenesis of hypertension as determined by a study of the effects of bilateral nephrectomy and other experimental procedures on the blood pressure of the dog. *Am. J. Physiol.*, **157**, 21–30.

HENSBY, C.N. & NAYLOR, B. (1974). The quantitative assay of prostaglandin $E_2$ and $F_{2\alpha}$ in biological extract using a Finnigan 3000D quadrupole mass spectrometer. *Br. J. Pharmac.*, **52**, 148P.

JAFFE, B.M., PARKER, C.W., MARSHALL, G.R. &

NEEDLEMAN, P. (1972). Renal concentration of PGE in acute and chronic renal ischaemia. *Biochem. biophys. Res. Commun.*, **49**, 799–805.

HERBACZYNSKA-CEDRO, K. & VANE, J.R. (1973). Contribution of intrarenal generation of prostaglandin to autoregulation of renal blood flow in dog. *Circulation Res.*, **33**, 428–436.

KOLFF, W.J. (1958). Reduction of experimental renal hypertension by kidney perfusion. *Circulation*, **17**, 702–707.

KRAMER, P. & OCHWADT, B. (1974). Separate renal concentrating ability in chronically hypertensive rats with unilateral Goldblatt clip. *Nephron*, **12**, 464–474.

LEARY, W.P., LEDINGHAM, J.G. & VANE, J.R. (1974). Imparied prostaglandin release from the kidneys of salt-loaded and hypertensive rats. *Prostaglandins*, **7**, 425–432.

LEE, J.B. (1973). Hypertension, natriuresis and the renomedullary prostaglandins. An overview. *Prostaglandins*, **3**, 551–579.

LONIGRO, A.J., TERRAGNO, N.A., MALIK, K.U. & McGIFF, J.C. (1973). Differential inhibition by prostaglandins of the renal actions of pressor stimuli. *Prostaglandins*, **3**, 595–606.

MALIK, K.U. & McGIFF, J.C. (1975). Modulation of prostaglandins of adrenergic transmission in the isolated perfused rabbit and rat kidney. *Circulation Res.*, **36**, 599–609.

McGIFF, J.C., CROWSHAW, K., TERRAGNO, R.A., LONIGRO, A.J., STRAND, J.C., WILLIAMSON, M.A., LEE, J.B. & NG, K.K.F. (1970). Prostaglandin-like substances appearing in the canine renal venous blood during renal ischaemia. *Circulation Res.*, **27**, 765–782.

McGIFF, J.C. & NASJLETTI, A. (1973). Renal prostaglandins and the regulation of blood pressure. In *Prostaglandins and cyclic AMP. Biological Actions and Clinical Applications*. ed. Kahl, R. & Lands, W. pp. 119–151. New York and London: Academic Press.

MERCER, P.F. (1971). Effects of bilateral vagotomy on renal function in the rat. *Can. J. Physiol. Pharmac.*, **49**, 420–426.

MUIRHEAD, E.E., GERMAIN, G.S., BROOKS, B. & STEPHEN, P. (1973). Renomedullary interstitial cells (RIC), prostaglandins (PG) and the antihypertensive function of the kidney. *Prostaglandins*, **3**, 581–594.

MUIRHEAD, E.E., LEACH, B.E., DANIELS, E.D. & HINMAN, J.W. (1968). Lapine renomedullary lipid in murine hypertension. *Arch. Path.*, **85**, 72–79.

MUIRHEAD, E.E., LEACH, B.E., GERMAIN, G.S., BYERS, L.W. & ARMSTRONG, F.B. (1974). Effect of indomethacin on antihypertensive lipids derived from renomedullary interstitial cells grown in tissue culture. *Fedn. Proc.*, **33**, 339.

OWEN, T., EHRHART, I., WEIDNER, J., HADDY, F. & SCOTT, J. (1974). Effects of indomethacin (I) on blood flow, reactive hyperaemia (RH) and autoregulation (AR) in the dog kidney. *Fedn. Proc.*, **33**, 348.

POYSER, N.L. (1972). Production of prostaglandins by the guinea-pig uterus. *J. Endocr.*, **54**, 147–159.

SIRVIOS, P. & GAGNON, D.J. (1974). Release of renomedullary prostaglandins in normal and hypertensive rats. *Experentia*, **30**, 1418–1419.

SOMOVA, L.I. (1971). A study of the vasodepressor lipid isolated from the kidneys of hypertensive animals. *Nephron*, **8**, 575–583.

SOMOVA, L.I. (1973). Inhibition of prostaglandin synthesis in the kidneys by aspirin-like drugs. *Adv. Biosci.*, **9**, 335–339.

THOMPSON, C.J., LOS, M. & HORTON, E.W. (1970). The separation, identification and estimation of prostaglandins in nanogram quantities by combined gas chromatography-mass spectrometry. *Life Sciences*, **9**, 983–988.

TOBIAN, L. & AZAR, S. (1971). Antihypertensive and other functions of the renal papilla. *Trans Am. Assoc. Physicians*, **84**, 281–288.

TOBIAN, L., SCHONNING, S. & SEEFELDT, C. (1964). The influence of arterial pressure on the antihypertensive action of a normal kidney, a biological servomechanism. *Ann. Intern. Med.*, **60**, 378–383.

VENUTO, R.C., O'DORISIO, T., FERRIS, T.F. & STEIN, J.H. (1975). Prostaglandins and renal function II. The effect of prostaglandin inhibition on autoregulation of blood flow in the intact kidney of dog. *Prostaglandins*, **9**, 817–828.

*(Received March 29, 1976.*
*Revised May 15, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 261–266

# THE LACK OF EFFECT OF OXYTETRACYCLINE ON RESPONSES TO SYMPATHETIC NERVE STIMULATION AND CATECHOLAMINES IN VASCULAR TISSUE

S. KALSNER
Department of Pharmacology, University of Ottawa, Ottawa, Ontario, Canada

1 The effects of oxytetracycline, an inhibitor of amine binding in connective tissue, on the responses of perfused rabbit ear arteries to sympathetic nerve stimulation and to intraluminally administered noradrenaline were examined. The contractions of aortic strips to catecholamines in the presence of oxytetracycline were also examined.

2 Oxytetracycline (0.1 mM) had no discernable effect on the magnitude of constrictions, measured as reductions in flow, produced by either nerve stimulation (0.5–10 Hz) or noradrenaline (0.5–50 ng) in the ear artery. In addition, the time taken for vessels to recover towards control flow values after endogenously released or exogenously applied noradrenaline had acted was not increased by oxytetracycline.

3 Oxytetracycline (0.1 mM) did not alter the position or shape of the concentration-response curve to noradrenaline nor did it enhance the amplitude of individual responses to catecholamines in aortic strips.

4 It is concluded, contrary to the observations of Powis (1973), that oxytetracycline does not increase the magnitude or duration of responses to sympathetic nerve activation or to catecholamines and that binding to connective tissue is of no material consequence in terminating their action in vascular tissue.

## Introduction

The recent report that responses of isolated blood vessels to sympathetic nerve stimulation and to pulses of injected noradrenaline are considerably enhanced and prolonged by oxytetracycline (Powis, 1973), presumably by blockade of catecholamine binding to collagen and elastin in the vascular wall, needs further investigation. If this finding is verifiable it severely complicates the analysis of termination of action of catecholamine-induced responses in vascular tissue, since it is not easily reconciled with the bulk of available evidence on agonist disposition. For this reason, experiments were undertaken to re-examine the effects of oxytetracycline on responses to nerve stimulation and to catecholamines in the central artery of the rabbit ear and to include, as well, observations on rabbit aortic tissue, which has an even greater amount of connective tissue than does the relatively muscular ear vessel.

## Methods

The central artery was removed from each ear in rabbits under urethane anaesthesia (7 ml/kg of a 25% solution, intraperitoneally) after cannulation at both ends, essentially as described by De La Lande & Rand (1965) and De La Lande, Frewin & Waterson (1967). The arteries were suspended in individual 30 ml muscle chambers containing Krebs-Henseleit (Krebs) solution of the following composition (mM): NaCl 115.3, KCl 4.6, $CaCl_2$ 2.3, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, $KH_2PO_4$ 1.1 and glucose 7.8, to which the disodium salt of ethylene diamine tetra-acetic acid was added (0.03 mM), to retard oxidation of catecholamines catalyzed by traces of heavy metals. The preparations were maintained at 37°C and continuously oxygenated with 95% $O_2$ and 5% $CO_2$.

A gravity-feed aparatus was used to perfuse the vessels at a constant pressure of 85 cm of water with warmed (37°C) and oxygenated Krebs solution. A record of the rate of flow of Krebs solution through each artery was provided by an Andrews (1952) outflow recorder connected to a piston recorder writing on a slowly revolving smoked kymograph drum. The height of each vertical stroke of the writing lever is proportional to the volume of air displaced from the outflow recorder over an 8 s period and thus to the volume of Krebs solution flowing through the vessel lumen and into the recorder during that time interval. Other details and a schematic diagram of the perfusion apparatus are presented elsewhere (Kalsner, 1972).

The procedure used for stimulation of the periarterial sympathetic nerves was that used by De La Lande & Rand (1965). Platinum electrodes were arranged around the proximal end of each artery over the area where the perfusion cannula lay within the artery. The nerves were stimulated at supramaximal voltage with biphasic pulses of 1 ms pulse width delivered by a Grass model S5 stimulator. Supramaximal voltage was determined for each preparation by stimulating initially at 5 Hz for 1 min at each test voltage (beginning with 10 V and increasing in 5 V increments) and recording the vasoconstriction produced. A voltage which was about 25% above that which produced the most vasoconstriction was routinely used in all experiments.

Strips of rabbit aorta were prepared and suspended under 2 g of tension in muscle chambers of 15 ml capacity (Kalsner & Nickerson, 1968). The strips were immersed in Krebs solution as described above, maintained at 37°C, and constantly bubbled with 95% $O_2$ and 5% $CO_2$. Isotonic contractions were recorded, after a 90 min equilibration period, by means of frontal-writing levers on a slowly moving kymograph drum (about 2.0 mm/min) with a lever magnification of 6.8-fold.

The drugs used were (−)-noradrenaline and (−)-adrenaline bitartrates and oxytetracycline dihydrate. The catecholamines are expressed as the weight of the base in g/ml and oxytetracycline is in terms of molarity. For intraluminal injections of noradrenaline in the ear arteries the desired absolute amount in ng was always delivered in a total volume of 0.2 ml, by injection into the tubing immediately above the vessel. The administration of 0.2 ml of Krebs solution, as a routine control measure, had no effect on flow through the artery. Oxytetracycline was dissolved directly into a reservoir of Krebs solution at a concentration of 0.1 mM shortly before use and the flow of drug-treated or control Krebs solution through the vessel was determined by means of a two-way stopcock located above the proximal end of the artery. In addition, Krebs solution containing oxytetracycline (0.1 mM) was added to the muscle chamber so as to bathe the extraluminal surface of the vessel. Preparations were exposed to the tetracycline compound, for 20 min before testing and for the entire test period. With respect to aortic strips, the oxytetracycline was either dissolved directly in the Krebs solution at 0.1 mM and the muscle chambers filled with the treated Krebs for 20 min, followed without washout, by agonist; or the tetracycline was made up in 0.9% w/v NaCl solution at a concentration of 2 mM, adjusted to pH 8.0 with 1 N NaOH, and the appropriate volume added to the muscle chambers after responses to the agonist had attained plateau values.

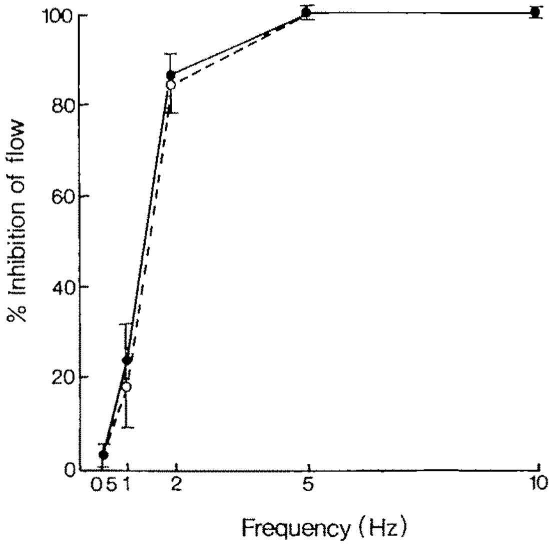


**Figure 1** Effect of oxytetracycline on the frequency-response curve to periarterial stimulation in the rabbit ear artery. Frequency-response curves in 7 preparations are shown, first in the absence (●) and then in the presence (○) of oxytetracycline (0.1 mM). Stimulation was for 2 min at each test frequency. Mean values are shown; vertical lines show s.e. mean. Details of test conditions are provided in the Methods section.

Response sensitivities in aortic strips at the mean effective dose ($ED_{50}$) are expressed as geometric mean values (Fleming, Westfall, De La Lande & Jellett, 1972). Each $ED_{50}$ was converted to its log and the mean for each group was recorded. The antilog of each mean log is presented as the geometric mean. In addition, mean values of all data are presented with their standard errors and were compared, where appropriate, by Student's *t* test.

## Results

### *Responses to nerve stimulation and to noradrenaline in the ear artery*

The central artery responds to periarterial stimulation of its sympathetic innervation with constriction, decreased lumen diameter and a consequent reduction in intraluminal flow. Detectable inhibition of flow is most often seen at 0.5–1.0 Hz and obliteration of lumen diameter and cessation of flow at 5 Hz. For the present experiments, two sets of frequency-response curves (0.5–10 Hz) were obtained for each vessel, one in the absence and the other in the presence of oxytetracycline (0.1 mM), so as to minimize variability.

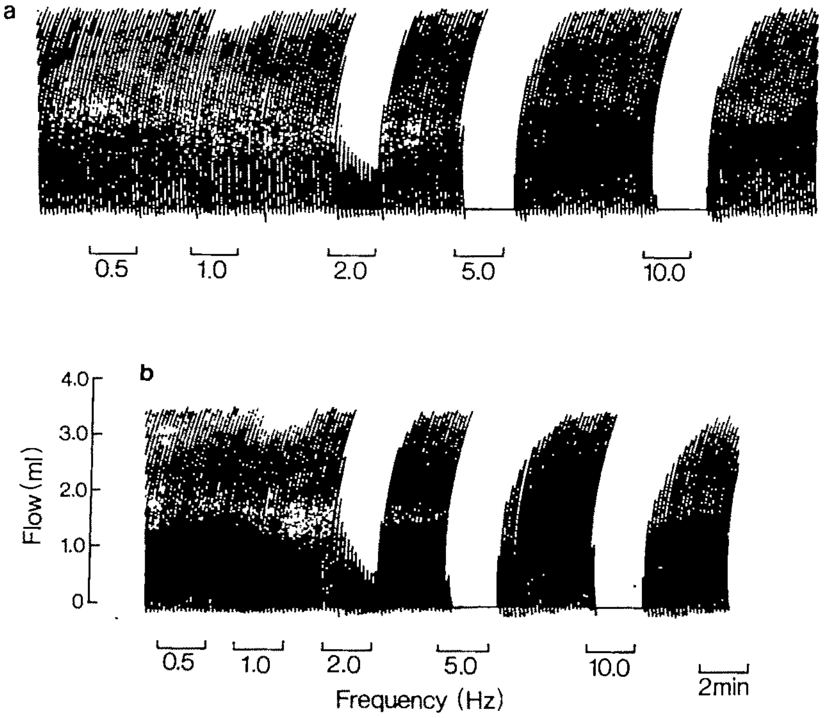


**Figure 2** Effect of oxytetracycline on responses of an ear artery to periarterial nerve stimulation. Responses of a typical vessel are shown (a) in the absence and (b) in the presence of oxytetracycline (0.1 mM). The duration of stimulation at each frequency was 2 min and is indicated by enclosed horizontal bars. Vasoconstrictor responses are recorded as reductions in outflow.

The cumulative results are shown in Figure 1 and the record of one such experiment is seen in Figure 2. No detectable effect of the tetracycline compound on the magnitude of flow inhibition at any of the tested frequencies was observed.

Additionally, analysis of response duration, measured as the time taken after the cessation of stimulation at 5 and 10 Hz, for flow to return to 50% of pre-stimulation values failed to reveal any statistically significant effect of the inhibitor in prolonging duration of action. The mean half-time for recovery in 7 control and 7 treated preparations was 33.4 ± 8.0 and 37.9 ± 8.0 s respectively at 5 Hz; and it was 39.0 ± 9.4 and 39.4 ± 9.0 s at 10 Hz. It was confirmed in matching experiments that, in the absence of oxytetracycline, first and second frequency-response curves do not differ significantly from each other either in the size or in the duration of the response.

Further, oxytetracycline (0.1 mM) had no enhancing effect on the constrictions elicited by intraluminal injections of noradrenaline (0.5–50 ng); a method of agonist administration similar to that which was used by Powis (1973). The dose-response curves obtained in the absence or in the presence of the inhibitor of amine binding did not differ significantly (Figure 3). Further, analysis of response duration again established the lack of interference by oxytetracycline with termination of action. The mean half-time for recovery from peak effects in 5 control and 5 treated preparations was 13.6 ± 1.7 and 15.2 ± 1.3 s respectively after 20 ng; and it was 46.0 ± 6.3 and 58.0 ± 10.9 s after 50 ng of noradrenaline.

### *Responses to noradrenaline and adrenaline in aortic strips*

Experiments were done on aortic strips using two different protocols. In one case, strips were contracted with low concentrations of noradrenaline (3 or 10 ng/ml) or adrenaline (10 ng/ml), chosen from the steep portions of the concentration-response curves, to maximize the size of any enhancing effect. After responses had reached a stable plateau value, oxytetracycline was added to the bathing fluid, either cumulatively (10, 30, 100 μM), or in one single addition (0.1 mM) (Figure 4). In a total of 8 strips

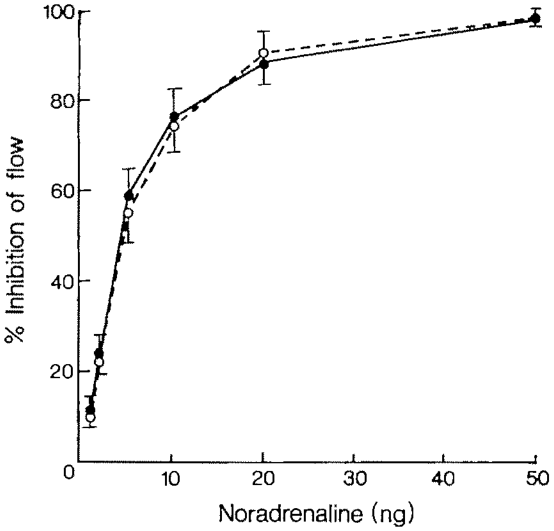


**Figure 3** Effect of oxytetracycline on responses of the ear artery to intraluminal injections of noradrenaline. Dose-response curves in 7 preparations, first in the absence (●) and then in the presence (○) of oxytetracycline (0.1 mM) are shown. Mean values are shown; vertical lines show s.e. mean. Details of agonist administration are given in the Methods section.

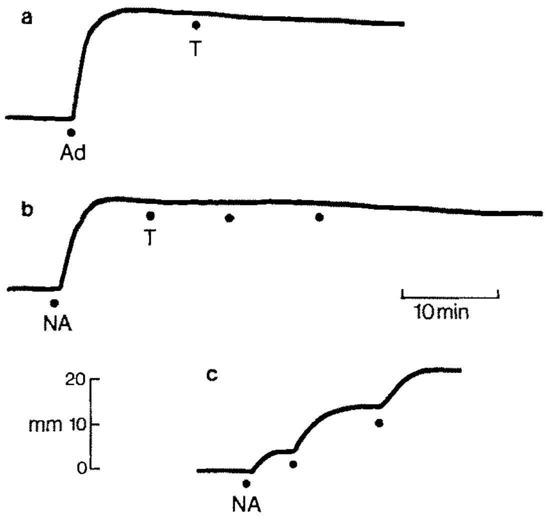


**Figure 4** Effect of oxytetracycline on responses of aortic strips to catecholamines. (a) Strip contracted by adrenaline (Ad, 10 ng) and exposed to oxytetracycline (T, 0.1 mM); (b) strip contracted by noradrenaline (NA, 3 ng) and exposed to oxytetracycline (T, 10, 30 and 100 µM at dots) (c), strip contracted cumulatively by noradrenaline (1, 3 and 10 ng) to show typical increments in amplitude obtained with approximately 3-fold increments in agonist concentration.

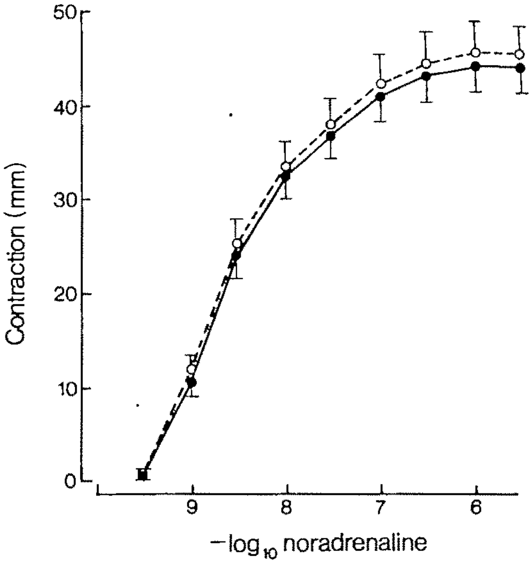


**Figure 5** Effect of oxytetracycline on the concentration-response curve to noradrenaline in aortic strips. Responses of 10 preparations in the absence (●) and 11 preparations in the presence (○) of oxytetracycline are shown. Mean values are shown; vertical lines show s.e. mean. Details are given in text.

examined no increment in response amplitude due to the oxytetracycline was detectable.

In the other case, strips were matched initially for responsiveness to noradrenaline by exposure to low concentrations (0.3, 1 and 3 ng/ml) and then after washout of agonist and recovery of basal tone half of the strips were exposed to oxytetracycline (0.1 mM) for 20 min and the remainder served as controls. Complete concentration-response curves to noradrenaline were then obtained in all strips, and this was done without washout of the oxytetracycline in the treated ones. The results are shown in Figure 5. Again, the postulated inhibitor of amine binding had no material effect on the magnitude of responses to noradrenaline, at any point along the curve. Also, geometric mean $ED_{50}$ values of the control and treated groups (2.8 and 2.6 ng/ml respectively), did not differ significantly ($P > 0.5$).

## Discussion

The observations of Powis (1973) on response potentiation by oxytetracycline could in no way be confirmed in the present experiments. Oxytetracycline, an inhibitor of amine binding to collagen and elastic tissue, neither sensitized the central ear artery to intraluminal injections of noradrenaline or to sympathetic nerve stimulation nor prolonged the duration of its responses. Similarly, aortic strips of the rabbit, rich in connective tissue, did not give increased contractions to noradrenaline in the presence of oxytetracycline; regardless of whether it was added before the catecholamine or after individual responses had reached stable equilibrium values.

Although the present findings cannot account for the enhanced contractions observed by Powis in the presence of oxytetracycline (in the order of 10- and 6-fold for responses to noradrenaline and nerve stimulation), certain apparent inconsistencies in his analysis should be mentioned. He concluded that in tissues with a high content of collagen and elastin 'binding to extracellular sites is the major mechanism for terminating the response to noradrenaline or to adrenergic nerve stimulation'. However, his data reveal that blockade of neuronal and effector cell uptake, with cocaine and 17β-oestradiol, produces a sensitization perhaps only slightly less than that caused by oxytetracycline (his Figure 4); but the addition of oxytetracycline to vessels already treated with cocaine and 17β-oestradiol does not produce any material additional enhancement. This is contrary to what would be expected if independent mechanisms of inactivation were inhibited together.

The suggestion that overlapping mechanisms of inactivation are involved such that binding to connective tissue is a prelude to definitive inactivation by neuronal or extraneuronal (effector cell) uptake is also not sustained by his data. For if, as he states, 'extracellular tissue binding is sufficient for the immediate inactivation of all the noradrenaline presented in a pulse injection, as employed in the present study, or liberated by nerve stimulation over a short period' then no demonstrable enhancing effect of 17β-oestradiol and cocaine, the inhibitors of effector cell and neuronal uptake respectively, should be observed under these conditions. And, enhancement was observed.

The explanation for the potentiating effect observed by Powis, which could not be duplicated here, is at present obscure. In this context, it should be noted that Guimarães, Azevedo, Cardoso & Oliveira (1975) did not detect enhancement by oxytetracycline of the inhibitory dose-response curves to isoproterenol in saphenous vein preparations of the dog.

A good deal of evidence obtained from various laboratories and by the use of several different techniques points to an important role for effector cell uptake, followed by enzymatic inactivation, in termination of action in most preparations of vascular tissue examined (Kalsner, 1966; Kalsner & Nickerson, 1969; Gillespie, Hamilton & Hosie, 1970; Berkowitz, Tarver & Spector, 1971; Bevan & Su, 1973; Kalsner, 1975a; Kalsner, Frew, Smith, 1975); although the role for neuronal uptake is still controversial (De La Lande, 1975; Kalsner, 1975b). Thus, although binding of catecholamine to connective tissue may occur, it is improbable that it could be an additional independent factor of any significance in termination of action, particularly with moderate concentrations of amine; unless our concept of biophase kinetics (Furchgott, 1955; Kalsner, 1976) is to be extensively revised. The available evidence does not warrant such a revision.

This research was supported by grants from the Ontario Heart Foundation and the Medical Research Council of Canada. I thank Mr M. Suleiman and Mr A. Driscoll for technical assistance.

## References

ANDREWS, W.H.H. (1952). A blood outflow recorder. *J. Physiol., Lond.*, **117**, 45–46P.

BERKOWITZ, B.A., TARVER, J.H. & SPECTOR, S. (1971). Norepinephrine in blood vessels: concentration, binding, uptake and depletion. *J. Pharmac. exp. Ther.*, **177**, 119–126.

BEVAN, J.A. & SU, C. (1973). Sympathetic mechanisms in blood vessels: nerve and muscle relationships. *Ann. Rev. Pharmac.*, **13**, 269–285.

DE LA LANDE, I.S. (1975). Adrenergic mechanisms in the rabbit ear artery: a review. *Blood Vessels*, **12**, 137–160.

DE LA LANDE, I.S., FREWIN, D. & WATERSON, G. (1967). The influence of sympathetic innervation on vascular sensitivity to noradrenaline. *Br. J. Pharmac.*, **31**, 82–93.

DE LA LANDE, I.S. & RAND, M.J. (1965). A simple isolated nerve-blood vessel preparation. *Aust. J. exp. Biol. Med. Sci.*, **43**, 639–656.

FLEMING, W.W., WESTFALL, D.P., DE LA LANDE, I.S. & JELLETT, L.B. (1972). Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. *J. Pharmac. exp. Ther.*, **181**, 339–345.

FURCHGOTT, R.F. (1955). Pharmacology of vascular smooth muscle. *Pharmac. Rev.*, **7**, 183–253.

GILLESPIE, J.S., HAMILTON, D.N.H. & HOSIE, R.J.A. (1970). The extraneuronal uptake and localization of noradrenaline in the cat spleen and the effect on this of some drugs, of cold and of denervation. *J. Physiol., Lond.*, **206**, 563–590.

GUIMARÃES, S., AZEVEDO, I., CARDOSO, W. & OLIVEIRA, M.C. (1975). Relation between the amount of smooth muscle of venous tissue and the degree of supersensitivity to isoprenaline caused by inhibition of catechol-O-methyl transferase. *Arch. Pharmac.*, **286**, 401–412.

KALSNER, S. (1966). Studies of the mechanisms terminating the action of the sympathetic nerve mediator. Ph.D. Thesis, University of Manitoba.

KALSNER, S. (1972). Effects of inhibition of noradrenaline uptake and synthesis on the maintenance of the response to continuous nerve stimulation in the central artery of the rabbit ear. *Br. J. Pharmac.*, **45**, 1–12.

KALSNER, S. (1975a). Role of extraneuronal mechanisms in the termination of contractile responses to amines in vascular tissue. *Br. J. Pharmac.*, **53**, 267–277.

KALSNER, S. (1975b). The importance of adrenergic neuronal uptake in termination of action: another view. *Blood Vessels*, **12**, 316–322.

KALSNER, S. (1976). Sensitization of effector responses by modification of agonist disposition mechanisms. *Can. J. Physiol. Pharmac.*, **54**, 177–187.

KALSNER, S., FREW, R.D. & SMITH, G.M. (1975). Mechanism of methylxanthine sensitization of norepinephrine responses in a coronary artery. *Am. J. Physiol.*, **228**, 1702–1707.

KALSNER, S. & NICKERSON, M. (1968). A method for the study of mechanisms of drug disposition in smooth muscle. *Can. J. Physiol. Pharmac.*, **46**, 719–730.

KALSNER, S. & NICKERSON, M. (1969). Effects of a haloalkylamine on responses to and disposition of sympathomimetic amines. *Br. J. Pharmac.*, **35**, 440–455.

POWIS, G. (1973). Binding of catecholamines to connective tissue and the effect upon the responses of blood vessels to noradrenaline and to nerve stimulation. *J. Physiol., Lond.*, **234**, 145–162.

*(Received April 5, 1976.)*

# PROCEEDINGS OF THE British Pharmacological Society

**15th–16th July, 1976**

UNIVERSITY OF DUNDEE

## COMMUNICATIONS

### Heterogeneity of inhibitory mechanisms in the nucleus accumbens and preoptic area of the rat

C.R. GARDNER & S.W. PHILLIPS

*Physiology Department, University of Cape Town, Observatory 7925 Cape Town.*

Microelectrode recordings of spontaneously active neurones in the nucleus accumbens and preoptic area of the urethane-anaesthetized rat showed similar responses to single square pulse stimulation (0.4–1.0 mA, 0.1–2.0 ms) of the amygdala or midbrain reticular formation. Responses were inhibitory or complex with an inhibitory component. Inhibition was either short (20–50 ms) or longer (100–300 ms).

Long inhibition was preceded by stimulus-locked action potentials (8–25 ms latency) in 36% ($n=56$) of observations but short inhibition was generally not preceded by excitation. Long inhibition may thus be due to activation of a recurrent pathway (Fenske, Ellendorff & Wuttke, 1975; Sakuma & Kawakami, 1975) whilst short inhibition may be a direct mechanism.

In order to determine whether these inhibitions are GABA-mediated, the effect of intravenous bicuculline (0.1–0.25 mg/kg) was investigated. Some cells showed cyclic changes in firing rate and when these were marked (>80%) the duration of inhibition, particularly that of long inhibition, was often altered. Thus, bicuculline-induced changes were considered with respect to changes in cell firing rate.

When a small or no change in firing rate occurred after bicuculline administration, the duration of long inhibition was reduced by 15–42% ($n=6$; Figure 1). However, the duration was increased if the firing rate was markedly raised, a change which was also observed in the absence of bicuculline.

Short inhibition was little affected by bicuculline unless the firing rate was markedly changed ($n=16$). Both types of inhibition could be evoked from the same neurone by stimulation at different sites and in these cases long inhibition was similarly reduced by bicuculline whilst short inhibition was little affected.

Thus long duration inhibition may be GABA-mediated but no evidence was obtained to support this view for short duration inhibition. It remains possible that short duration inhibition may result from the release of another transmitter.

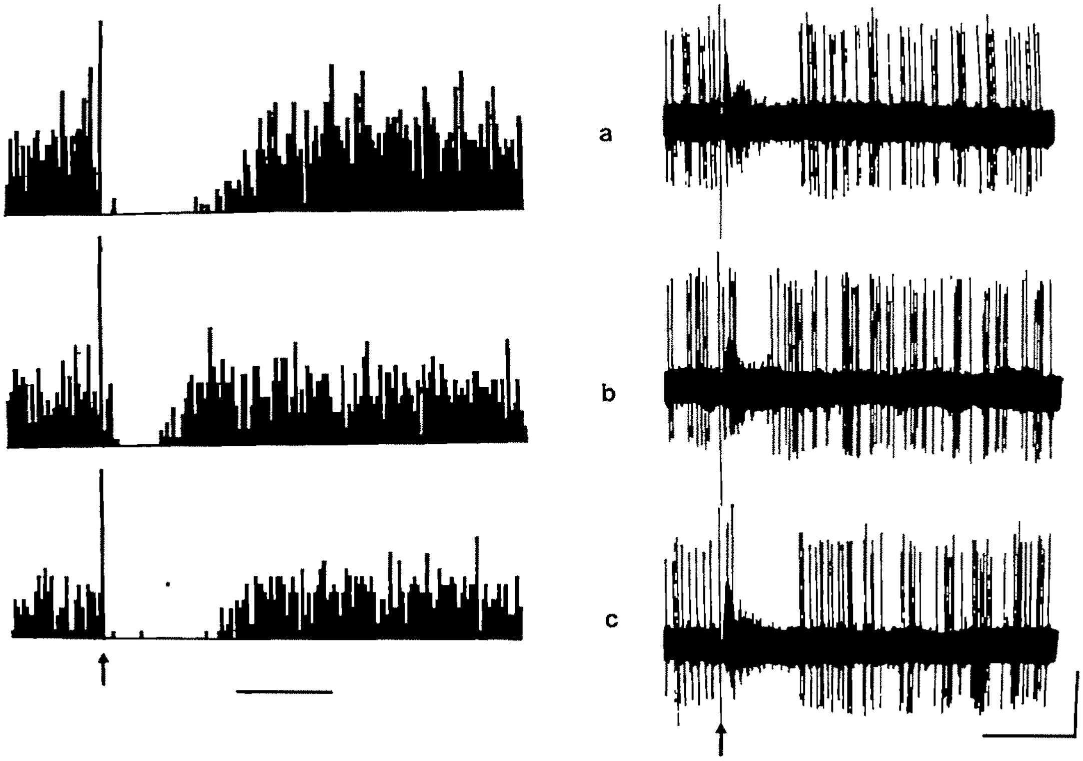


**Figure 1** The effect of bicuculline (0.1 mg/kg i.v.) on long duration inhibition in the preoptic area of a urethane-anaesthetized rat. Each pair of traces shows a post-stimulus-times histogram constructed from responses to 200 single stimuli delivered in the amygdala (left) and ten superimposed oscilloscope sweeps of action potential traces (right). The position of the stimulus is indicated by the arrow. (a) Control responses. (b) Responses recorded during the action of bicuculline. The histogram was constructed from responses in the period 10–110 s after drug administration. The spike records were taken 30–40 s after bicuculline. (c) Recovery 20–22 min after bicuculline.
Time calibrations—100 ms. Voltage calibration—100 μV.

## References

FENSKE, M., ELLENDORFF, F. & WUTTKE, W. (1975). Responses of medial preoptic neurons to electrical stimulation of the mediobasal hypothalamus, amygdala and mesencephalon in normal, serotonin or catecholamine deprived female rats. *Exp. Brain Res.*, **22**, 495–507.

SAKUMA, Y. & KAWAKAMI, M. (1975). Neural and humoral interactions between basal prechiasmatic area and median eminence. In: *Progress in Brain Research*, **42**, eds. Gispen, W.M., van Wimersma Greidanus, Tj.B., Bohus, B. and De Wied, D., p. 323–324. Elsevier, Amsterdam.

## Comparative studies on the effects of metoclopramide and some known neuroleptics on the EEG of the conscious rat

M.G. BAXTER, A.A. MILLER & P.L. WHEATLEY

*Pharmacology Laboratory, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS*

Like the clinically used neuroleptics, metoclopramide, an anti-emetic, when given to laboratory animals induces catalepsy (Ahtee, 1975), antagonizes apomorphine-induced stereotypy and amphetamine-induced ipsiversive circling (Dolphin, Jenner, Marsden, Pycock & Tarsy, 1975) and increases brain homovanillic acid concentrations (Peringer, Jenner & Marsden, 1975). However, it has apparently little neuroleptic action in man (Borenstein & Bles, 1965). The predictive value of these animal tests being in question we considered it of interest to compare metoclopramide with three clinically used neuroleptics (chlorpromazine, haloperidol and pimozide), with regard to their effect on the electroencephalogram (EEG) and on dexamphetamine-induced alerting of the EEG in the conscious rat.

EEG studies were undertaken in rats (male, 250 to 350 g) chronically implanted with skull electrodes (Goff, Miller, Smith, Smith and Wheatley, 1975). The parietal EEG, recorded on magnetic tape was analysed for 10 s periods at 10 s intervals by passing it through four broad wave band filters which measured the voltage within each of the following frequency bands (FB): 2.4 to 4.0, 4.0 to 7.5, 7.5 to 13.5 and 13.5 to 26.0 Hz (FB1 to FB4 respectively). The hourly mean integrated voltages were calculated. The neuroleptics were injected subcutaneously at the ED$_{95}$ values found to antagonize the stereotypy induced 30 min after apomorphine (5 mg/kg s.c.) in tests in which they were administered 60 min previously; similar doses antagonized dexamphetamine-induced (5 mg/kg s.c.) stereotypy.

In the EEG studies with drugs alone the mean hourly voltages in the 4 h period after drug administration were compared with the mean value for the preceding 2 h control period. Chlorpromazine (8.4 mg/kg, $n=3$) increased ($P\leqslant 0.05$) total voltage and voltages in each FB at all times. Haloperidol (1.3 mg/kg, $n=3$) increased ($P\leqslant 0.05$) total FB3 and FB4 voltages from 2 to 4 h but metoclopramide (17.0 mg/kg, $n=3$) and pimozide (1.7 mg/kg, $n=3$) like the control vehicle ($n=9$), had no effect.

In the EEG studies of dexamphetamine-induced alerting, the neuroleptics, or control vehicle, were injected at the end of a 2 h control period and 1 h before dexamphetamine (0.3 mg/kg s.c.). In control rats ($n=5$) this dose of dexamphetamine had no gross behavioural effects but showed a significant reduction in total voltage for the first hour only ($P=0.05$). This alerting effect was antagonized by chlorpromazine, haloperidol and pimozide ($n=3$ or 4) but was prolonged by metoclopramide ($n=5$) with voltage being significantly reduced at both 1 and 2 h ($P\leqslant 0.03$).

Dexamphetamine's altering action may be attributable to noradrenaline release in the brain stem (Boakes, Bradley & Candy, 1972) and its antagonism by chlorpromazine and haloperidol may reflect their ability to block noradrenaline receptors in addition to their blockade of dopamine receptors (Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970). However, there is no obvious correlation between the effects of the neuroleptics on dexamphetamine-induced EEG alerting and their ability to block catecholamine receptors as both pimozide and metoclopramide which block dopamine but not noradrenaline (Andén *et al.*, 1970; Peringer *et al.*, 1975) had contrasting actions. The potentiation of dexamphetamine by metoclopramide may be explained by inhibition of dexamphetamine or noradrenaline metabolism.

Additionally, our studies suggest that tests for blockade of dexamphetamine-induced EEG alerting may perhaps be of more value than others in predicting clinical usefulness of neuroleptics.

### References

AHTEE, L. (1975). Inhibition by apomorphine of the metoclopramide-induced catalepsy and increase in striatal homovanillic acid content. *Br. J. Pharmac.* **55**, 381–385.

ANDÉN, N., BUTCHER, S.G., CORRODI, H., FUXE, K. & UNGERSTEDT, U. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmac.* **11**, 303–314.

BOAKES, R.J., BRADLEY, P.B. & CANDY, J.M. (1972). A neuronal basis for the alerting action of (+)-amphetamine. *Br. J. Pharmac.* **45**, 391–403.

BORENSTEIN, P. & BLES, G. (1965). Effets cliniques et électroencéphalographiques du métoclopramide en psychiatrie. *Thérapie*, **20**, 975–995.

DOLPHIN, A., JENNER, P., MARSDEN, C.D., PYCOCK, C. & TARSY, D. (1975). Pharmacological evidence for cerebral dopamine receptor blockade by metoclopramide in rodents. *Psychopharmacologia (Berl.)*, **41**, 133–138.

GOFF, D., MILLER, A.A., SMITH, R.E., SMITH, S.J. & WHEATLEY, P.L. (1975). Combined EEG recording and intraventricular administration of drugs in the conscious rat. *Br. J. Pharmac.* **55**, 312–313P.

PERINGER, E., JENNER, P. & MARSDEN, C.D. (1975). Effect of metoclopramide on turnover of brain dopamine, noradrenaline and 5-hydroxytryptamine. *J. Pharm. Pharmac.* **27**, 442–444.

# Comparison of the self-administration liabilities of morphine and pentazocine in the rhesus monkey

R.C. HILL & D. RÖMER

*Biological and Medical Research Division, Sandoz Ltd., Basle, Switzerland*

A comparison of the self-administration liability of morphine hydrochloride and pentazocine was made in 2 male (approx. 3.0 kg), drug-experienced rhesus monkeys using a method based on that described by Deneau, Yanagita & Seever (1969). The experiment is summarized in Table 1. The number of lever presses made and the i.v. injections received by each monkey were registered automatically and the totals for each daily 23 h session were recorded. Both animals were kept under continuous observation for 10 h daily (7 a.m.–5 p.m.) with the aid of closed circuit television.

**Table 1** Mean number of lever presses made and i.v. injections received daily by 2 male rhesus monkeys in periods of access to morphine hydrochloride (0.1 mg/kg per injection) and pentazocine (0.032 and 0.1 mg/kg per injection).

| *Test substance* | *Dose per injection mg/kg i.v.* | *Week No.* | *Daily mean values* | | *Mean daily dose mg/kg i.v.* |
|---|---|---|---|---|---|
| | | | *Lever presses* | *Injections* | |
| Morphine HCl | 0.1 | 1 | 59 | 49 | 4.9 |
| | 0.1 | 2 | 62 | 56 | 5.6 |
| | 0.1 | 3 | 74 | 62 | 6.2 |
| | 0.1 | 4 | 132 | 117 | 11.7 |
| Saline | — | 5 | Marked withdrawal syndrome | | |
| Pentazocine | 0.032 | 6 | 297 | 229 | 7.3 |
| | 0.032 | 7 | 340 | 252 | 8.1 |
| | 0.032 | 8 | 444 | 354 | 11.3 |
| | 0.032 | 9 | 545 | 426 | 13.6 |
| Pentazocine | 0.1 | 10 | 396 | 358 | 35.8 |
| | 0.1 | 11 | 440 | 413 | 41.3 |
| Saline | — | 12 | Mild withdrawal syndrome | | |

The monkeys self-administered both substances, the mean daily dose increasing with time (Table 1). Naloxone hydrochloride given at intervals during the last week of access to morphine HCl (0.1 mg/kg i.v.) and pentazocine (0.1 mg/kg i.v.) produced short-lasting (maximum 2 h), opiate-like withdrawal syndromes. In animals administering morphine, naloxone (0.0032–0.032 mg/kg i.v.) produced a dose dependent increase in the duration, intensity and variety of the withdrawal signs and saline substitution resulted in a marked withdrawal syndrome lasting 3 days and accompanied initially by a 4-fold increase in lever pressing activity. During the pentazocine access period, naloxone (0.1–10 mg/kg i.v.) produced a mild, non-dose related withdrawal syndrome, saline substitution resulting in a mild withdrawal syndrome with no increase in lever pressing activity.

The results indicate that pentazocine produced a mild degree of physical dependence that was quantitatively and in some aspects qualitatively different to that exhibited by morphine. Also, the difference in the rates of lever pressing observed during saline substitution for both drugs suggests that pentazocine may not have the same ability as morphine to cause psychological dependence.

We thank Sterling Drug Inc. for the donation of pentazocine (Talwin).

## Reference

DENEAU, G., YANAGITA, T. & SEEVERS, M.H. (1969). Self-administration of psychoactive substances by the monkey. *Psychopharmacologia (Berl.)*, **16**, 30–48.

## Some effects of mazindol on the metabolism of monoamines in the rat brain

K.G. CHARLTON & M.F. SUGRUE

*Department of Pharmacology, Organon Scientific Development Group, Organon Laboratories Ltd., Newhouse, Lanarkshire ML1 5SH.*

Animal and human studies have shown mazindol (5-hydroxy-5-(4′-chlorophenyl)-2,3-dihydro-5H-imidazo-(2,1-a)isoindole) to be an effective anorectic agent (Goldrick, Nestel & Havenstein, 1974; Gogerty, Penberthy, Iorio & Trapold, 1975; Haugen, 1975). Brain monoamines have been implicated in the mechanism of action of anorectic drugs. For example, it has been proposed that 5-hydroxytryptamine and catecholamines play an important role in the anorectic effect of fenfluramine and (+)-amphetamine respectively (Garattini, Bizzi, de Gaetano, Jori & Samanin, 1975). The objective of this study was to investigate some of the effects of mazindol on the metabolism of 5-hydroxytryptamine, noradrenaline and dopamine in the rat brain.

Male Wistar rats weighing 180–220 g were used in all studies. Mazindol (30 mg/kg, i.p.) had no effect on steady state levels of 5-hydroxytryptamine, 5-hydroxyindole acetic acid or tryptophan in the rat brain at 1,2 and 4 h after injection. The turnover of 5-hydroxytryptamine in the brain was also unaltered by mazindol. The *in vivo* blockade of rat brain 5-hydroxytryptamine re-uptake was studied by investigating the effect of drug pretreatment on the ability of *p*-chloroamphetamine (10 mg/kg, i.p.) to lower brain 5-hydroxytrytamine content. Unlike compounds which block 5-hydroxytryptamine re-uptake, e.g. the tricyclic antidepressants, mazindol (30 mg/kg, i.p.) did not antagonize the *p*-chloroamphetamine induced reduction in the concentration of brain 5-hydroxytryptamine.

Mazindol (30 mg/kg, i.p.) evoked a slight but significant decrease in rat brain steady state levels of both noradrenaline and dopamine. *In vivo* central catecholamine re-uptake was studied by investigating the effect of drug pretreatment on the ability of intraventricularly administered 6-hydroxydopamine to lower brain levels of noradrenaline and dopamine. Both mazindol and desipramine antagonized the ability of intraventricularly administered 6-hydroxydopamine (100 μg) to lower brain steady state levels of noradrenaline. Mazindol was approximately five times more potent than desipramine. The ability of intraventricularly administered 6-hydroxydopamine (250 μg) to lower rat brain dopamine content was unaltered by the prior administration of desipramine (30 mg/kg, i.p.). In contrast to desipramine, mazindol was a potent inhibitor of the 6-hydroxydopamine induced fall in brain dopamine concentration, the i.p. $ID_{50}$ value for mazindol being 10.3 mg/kg. In addition to blocking dopamine re-uptake, mazindol like (+)-amphetamine (Dorris & Shore, 1974), also released dopamine as indicated by its ability to lower levels of $\alpha$-methyl-*m*-tyramine in the rat corpus striatum.

The results of this study reveal that mazindol is a potent inhibitor of the *in vivo* re-uptake of both noradrenaline and dopamine in the rat brain. In addition to blocking dopamine re-uptake, mazindol also releases the amine. In contrast to its marked effects on brain catecholaminergic systems, mazindol would appear to be devoid of effect on rat brain serotoninergic systems. The neurochemical profile of mazindol bears a much closer resemblance to that of (+)-amphetamine than to that of fenfluramine.

### References

DORRIS, R.L. & SHORE, P.A. (1974). Interaction of apomorphine, neuroleptics and stimulants with $\alpha$-methyl-*m*-tyramine, a false dopaminergic transmitter. *Biochem. Pharmac.*, **23**, 867–872.

GARATTINI, S., BIZZI, A., DE GAETANO, G., JORI, A. & SAMANIN, R. (1975). Recent advances in the pharmacology of anorectic agents. In: *Recent Advances in Obesity Research*, **1**, ed. Howard, A., pp. 354–367. London: Newman.

GOGERTY, J.H., PENBERTHY, C., IORIO, L.C. & TRAPOLD, J.H. (1975). Pharmacological analysis of a new anorexic substance: 5-hydroxy-5-(4′-chlorophenyl)-2,3-dihydro-5H-imidazo(2,1-a) isoindole (mazindol). *Arch. int. Pharmacodyn. Thér.*, **214**, 285–307.

GOLDRICK, R.B., NESTEL, P.J. & HAVENSTEIN, N. (1974). Comparison of a new anorectic agent AN448 with fenfluramine in the treatment of refractory obesity. *Medical Journal of Australia*, **1**, 882–885.

HAUGEN, H.N. (1975). Double blind cross-over study of a new appetite suppressant AN 448. *Eur. J. Clin. Pharmac.*, **8**, 71–74.

## Is the striato-nigral pathway responsible for 'feed-back' control of dopamine release?

G.W. ARBUTHNOTT, M. GARCIA-MUNOZ, N.M. NICOLAOU, I.F. TULLOCH & A.K. WRIGHT (introduced by G.W. ASHCROFT)

*M.R. C. Brain Metabolism Unit, University Department of Pharmacology, Edinburgh*

Blockade of post-synaptic dopamine (DA) receptors by haloperidol and similar neuroleptics is thought to increase DA turnover via a neuronal feedback pathway. The most obvious candidate for this function is the gabaminergic pathway from striatum to substantia nigra.

The pathway followed by the striato-nigral neurones was traced autoradiographically after [$^3$H]-leucine injection into striatum following the methods of Cowan, Gottlieb, Hendrickson, Price & Woolsey (1972). At the level of the mamillary bodies this pathway lies in the internal capsule more lateral than the ascending axons of the nigro-striatal system.

Electrolytic lesions of the striato-nigral pathway were made which completely spared the dopamine containing nigro-striatal projection. Rats with such lesions turned towards the lesioned side after i.p. apomorphine (0.5–5.0 mg/kg) and amphetamine (2 mg/kg). These animals behave like animals with large unilateral lesions of the striatum (Andén, Dahlström, Fuxe, Larsson, 1966) although they have normal levels of dopamine and its acid metabolites on both sides of the brain.

If the pathway from striatum to substantia nigra is involved in the response of the dopamine neurones to neuroleptics then the lesion should reduce the rise in homovanillic acid (HVA) 3,4,-dihydroxyphenylacetic acid (DOPAC) in the striatum after haloperidol administration. The level of DA was estimated by the methods of Coyle and Henry (1973), and HVA and DOPAC by a modification of the method of Pearson & Sharman (1975a), in single striata from rats injected with haloperidol (1 mg/kg i.p.) 30 min before they were killed. The concentration of HVA increased more than 500% on both sides in animals with unilateral lesions in the striato-nigral pathway. The increase in DOPAC (300%) and the decrease in DA (50%) were also similar in lesioned and unlesioned striata.

That the lesions were effective is indicated by a 60% fall in the levels of GABA in substantia nigra on the lesioned side estimated by a modification of the method of Pearson and Sharman (1975b) although the levels of DA were not different.

In conclusion it seems unlikely that a feed-back via the striato-nigral GABA containing neurones can be responsible for the effect of haloperidol on DA release.

### References

ANDÉN, N.E., DAHLSTRÖM, A., FUXE, K. & LARSSON, K. (1966). Functional role of nigro-neostriatal dopamine neurones. *Acta pharmac. Tox.*, **24**, 263–274.

COWAN, W.M., GOTTLIEB, D.I., HENDRICKSON, A.E., PRICE, J.L. & WOOLSEY, T.A. (1972). The autoradiographic demonstration of axonal connections in the central nervous system. *Brain Res.*, **37**, 21–51.

COYLE, J.T. & HENRY, D. (1973). Catecholamines in fetal and newborn rat brain. *J. Neurochem.*, **21**, 61–67.

PEARSON, J.D.M. & SHARMAN, D.F. (1975a). The estimation of 3,4-dihydroxyphenylacetic acid, homovanillic acid and homo-isovanillic acid in nervous tissue by gas-liquid chromatography and electron capture detection. *Br. J. Pharmac.*, **53**, 143–148.

PEARSON, J.D.M. & SHARMAN, D.F. (1975). A method for the estimation of γ-aminobutyric acid using gas-liquid chromatography and electron capture detection. *J. Neurochem.*, **24**, 1225–1228.

---

## The effects of anorectic drugs on uptake and release of brain monoamines

Z.L. KRUK & M.R. ZARRINDAST

*Department of Pharmacology and Therapeutics, The London Hospital Medical College, Turner Street, London, E1 2AD.*

While it is generally agreed that the action of anorectic drugs in the CNS involves catecholamines and 5-hydroxytryptamine (5-HT), it is still not clearly established which neurochemical systems are affected by individual anorectic drugs. As many anorectic drugs have some indirect sympathomimetic activity it was decided to examine the ability of these compounds to block the uptake or to facilitate the release of dopamine, noradrenaline and 5-HT from brain tissues.

Uptake was measured by the method of Snyder & Coyle (1969), and release was measured by the method of Ferris, Tang & Maxwell (1972), using tritiated monoamines and synaptosomes prepared from rat brain regions rich in nerve terminals containing those monoamines.

**Table 1** The effect of anorectic drugs on uptake and release of tritiated monoamines measured in synaptosomes prepared from the brain region indicated. The number of determinations made is indicated in the brackets.

| | *$^3$H-Dopamine (Striatum)* | | *$^3$H-5-Hydroxytryptamine (Striatum)* | | *$^3$H-Noradrenaline (Hypothalamus)* |
|---|---|---|---|---|---|
| *Compound* | *$IC_{50}$/(μM)* | *$RC_{50}$/(μM)* | *$IC_{50}$/(μM)* | *$RC_{50}$/(μM)* | *$IC_{50}$/(μM)* |
| D-Amphetamine | 0.05 ± 0.007<br>(8) | 0.25 ± 0.22<br>(6) | 1.2 ± 0.3<br>(6) | 3.0 ± 1.3<br>(5) | 0.07 ± 0.006<br>(6) |
| Phenmetrazine | 0.1 ± 0.04<br>(5) | 10.0 ± 1.0<br>(4) | 7.0 ± 1.0<br>(5) | 80.0 ± 18.0<br>(5) | 0.4 ± 0.06<br>(5) |
| Phentermine | 0.23 ± 0.05<br>(4) | 1.4 ± 1.3<br>(6) | 1.4 ± 0.1<br>(4) | 22.0 ± 13.0<br>(5) | 0.4 ± 0.02<br>(5) |
| Mazindol | 0.25 ± 0.04<br>(6) | 44.0 ± 4.0<br>(6) | 0.16 ± 0.06<br>(8) | 90.0 ± 44.0<br>(5) | 0.004 ± 0.004<br>(9) |
| Diethylpropion | 120.0 ± 20.0<br>(4) | 56.0 ± 3.0<br>(6) | ($>10^{-4}$ M) | ($>10^{-4}$ M) | 16.0 ± 4.0<br>(4) |
| p-Chloramphetamine | 0.16 ± 0.02<br>(5) | 2.3 ± 0.1<br>(5) | 0.02 ± 0.007<br>(5) | 0.13 ± 0.04<br>(5) | 0.5 ± 0.06<br>(6) |
| Norfenfluramine | 1.7 ± 0.8<br>(3) | 0.56 ± 0.17<br>(4) | 0.24 ± 1.0<br>(5) | 0.58 ± 0.05<br>(5) | 0.5 ± 0.11<br>(5) |
| Chlorphentermine | 6.0 ± 0.2<br>(4) | 9.5 ± 1.0<br>(4) | 0.085 ± 0.022<br>(5) | 0.16 ± 0.07<br>(5) | 0.9 ± 0.12<br>(5) |
| Fenfluramine | 12.0 ± 3.0<br>(4) | 34.0 ± 3.0<br>(4) | 0.3 ± 0.13<br>(5) | 0.65 ± 0.18<br>(5) | 1.8 ± 0.8<br>(4) |

$IC_{50}$ and $RC_{50}$ are the molar concentrations causing 50% inhibition of monoamine uptake and release respectively.

The results are expressed as $IC_{50}$ and $RC_{50}$ values, defined as the molar concentration of drug causing 50% inhibition of uptake, or 50% release of monoamine respectively. These values were derived from graphs in which % inhibition of uptake or % release was plotted against log molar concentration of anorectic drug.

The drugs examined were generally more potent as inhibitors of uptake than as releasers of monoamines. All the drugs examined were weak releasers of noradrenaline with $RC_{50} > 10^{-4}$ M.

Dexamphetamine, mazindol (5-hydroxy-5-(4′-chlorophenyl)-2,3-dihydro-5H-imidazo(2,1-a) isoindole), phentermine, phenmetrazine and diethylpropion were more potent in affecting uptake and release of dopamine than 5-HT. Fenfluramine, norfenfluramine, chlorphentermine and p-chloraphetamine were more potent in affecting release and uptake of 5-HT than dopamine. These results confirm that anorectic drugs act through either a dopaminergic or 5-HT mechanism in the brain (Kruk, 1973; Clineschmidt, McGuffin & Werner, 1974) and that noradrenergic mechanisms are unlikely to have a significant role in mediating anorectic responses.

### References

CLINESCHMIDT, B.V., McGUFFIN, J.C. & WERNER, B.A. (1974). Role of monoamines in the anorexigenic actions of fenfluramine, amphetamine and p-chloromethylamphetamine. *Eur. J. Pharmac.*, **27**, 313–323.

FERRIS, R.M., TANG, F.L.M. & MAXWELL, R.A. (1972). A comparison of the capacities of isomers of amphetamine, deoxypipradol and methylphenidate to inhibit the uptake of tritiated catecholamines into rat cerebral cortex slices, synaptosomal preparations and rat cerebral cortex, hypothalamus and striatum and into adrenergic nerves of rabbit aorta. *J. Pharmac. exp. Ther.*, **181**, 407–416.

KRUK, Z.L. (1973). Dopamine and 5-Hydroxytryptamine inhibit feeding in rats. *Nature, New Biol.*, **246**, 52–53.

SNYDER, S.H. & COYLE, J.T. (1969). Regional differences in $^3$H-norepinephrine and $^3$H-dopamine uptake into rat brain homogenates. *J. Pharmac. exp. Ther.*, **165**, 78–86.

# Effect of chronic pretreatment with sympathomimetic bronchodilator drugs on the responsiveness of guinea-pig lung adenylate cyclase *in vitro*

M.S. ELFELLAH, P.B. MARSHALL & M.J. TURNBULL[1]

*Department of Pharmacology, University of Dundee, Dundee, Scotland.*

Tolerance to the cardiac stimulatory and bronchodilator effect of the sympathomimetic drugs used in the treatment of asthma has been shown to develop in man and the guinea-pig respectively (Conolly, Davies, Dollery & George, 1971; Bouhuys, Douglas & Lewis, 1972; Benoy, Elfellah, Schneider & Wade, 1975). However, the mechanism(s) by which such tolerance is brought about is unknown. One possibility is that the drugs used can desensitize the $\beta$-adrenoceptors. Since there is strong evidence to support the hypothesis that $\beta$-adrenoceptors are associated with adenylate cyclase (Robison, Butcher & Sutherland, 1971), we have investigated the effect of pretreatment with sympathomimetic bronchodilator drugs on guinea-pig adenylate cyclase activity *in vitro*.

The animals were pretreated as previously described (Benoy *et al.*, 1975), killed by cervical dislocation and lung slices (1 mm in thickness) prepared using a McIlwain tissue chopper. In some experiments, tracheal rings were also used. Incubation conditions and methods were as described by Kakiuchi & Rall (1968) with the following modifications: the incubation was terminated by immersing the tubes in a boiling water bath for 3 min and homogenates of the tissue were prepared in the incubation medium using a Silverson homogenizer. One ml aliquots were removed for protein determination (Lowry, Rosebrough, Farr & Randall, 1951) and the remaining solution was centrifuged at 2000 *g* for 10 min and used for the assay of cyclic AMP using the method of Gilman (1970) as modified by Torey, Oldham & Whelan (1974).

There was no change in basal adenylate cyclase activity in either lung slices or tracheal rings after any drug pretreatment. However, after pretreatment of animals with isoprenaline for 7 days, but not for 3 days, with thrice daily s.c. injections of 5 µg/kg, the sensitivity of adenylate cyclase to isoprenaline ($10^{-4}$ M) was reduced in lung slices [control 173 ± 25 (10); pretreated 86 ± 12 (10) ($P < 0.01$); results depict mean activity expressed as pmol cAMP.mg protein$^{-1}$ 6 min$^{-1}$ minus basal activity ± s.e. mean] and also in tracheal rings [control 24 ± 6 (6); pretreated 8 ± 3 (6) ($P < 0.01$); results expressed as pmol cAMP tracheal ring$^{-6}$ 6 min$^{-1}$]. In animals similarly pretreated with isoprenaline, cross-tolerance was shown in lung slices challenged with noradrenaline ($5 \times 10^{-5}$ M) [Control 130 ± 20 (4); pretreated 52 ± 8 (4) ($P < 0.02$)] but not when challenged with prostaglandin $E_1$ ($5 \times 10^{-6}$ M) [control 387 ± 28 (6); pretreated 340 ± 50 (6)].

When guinea-pigs were pretreated with salbutamol (thrice daily s.c. injections of 10 µg/kg) a cross-tolerance developed to isoprenaline, but not to prostaglandin $E_1$, in lung slices and tracheal rings.

Since we have previously found (Elfellah and Turnbull—to be published) that lung phosphodiesterase activity is unaffected by the above drug pretreatments, our results suggest that the reduced sensitivity of adenylate cyclase found in the present experiments may be due to a desensitization of the enzyme caused by the prior *in vivo* exposure to the bronchodilator drugs. It is tempting to speculate that this phenomenon may be related to the development of tolerance to bronchodilator drugs in asthmatic subjects.

The authors wish to thank Mr A. Reid for technical assistance and the University of Benghazi, Libya, for financial support. We are grateful to Dr Pike of the Upjohn Co., Kalamazoo, for the sample of $PGE_1$.

**References**

BENOY, C.T., ELFELLAH, M.S., SCHNEIDER, R. & WADE, O.L. (1975). Tolerance to sympathomimetic bronchodilators in guinea-pig isolated lungs following chronic administration *in vivo*. *Br. J. Pharmac.*, **55**, 547–555.

BOUHUYS, A., DOUGLAS, J.S. & LEWIS, A.J. (1972). Hypersensitivity to adrenoceptor agents in the guinea-pig *in vitro* and *in vivo*. *Br. J. Pharmac.*, **46**, 520–522P.

CONOLLY, M.E., DAVIES, D.S., DOLLERY, C.T. & GEORGE, C.F. (1971). Resistance to $\beta$-adrenoceptor stimulation (a possible explanation for the rise in asthma deaths). *Br. J. Pharmac.*, **43**, 389–402.

GILMAN, A. (1970). A protein binding assay for adenosine 3′,5′ cyclic monophosphate. *Proc. natn. Acad. Sci. U.S.A.*, **67**, 305–312.

KAKIUCHI, S. & RALL, T.W. (1968). The influence of chemical agents on the accumulation of adenosine 3′,5′ phosphate in slices of rabbit cerebellum. *Molec. Pharmac.*, **4**, 367–378.

LOWRY, D.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

ROBISON, G.A., BUTCHER, R.W. & SUTHERLAND, E.W. (1971). See *Cyclic AMP*. New York and London: Academic Press.

TOREY, K.C., OLDHAM, K.G. & WHELAN, J.A.A. (1974). A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. *Clinica chim. Acta*, **56**, 221–234.

---

[1] Present address: ICI Ltd., Alderley Park, Macclesfield, Cheshire.

## The effect of acute doses of buprenorphine on concentrations of homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) in the rat forebrain

A. COWAN, P.W. DETTMAR & D.S. WALTER

*Department of Pharmacology, Reckitt & Colman, Kingston-upon-Hull HU8 7DS*

The new narcotic antagonist analgesic, buprenorphine (0.01–1.0 mg/kg, s.c.), induces ipsilateral turning behaviour in rats with lesions (caused by 6-hydroxydopamine) in the left substantia nigra (Cowan, Dettmar & Walter, 1975a). Over the same dose range, buprenorphine antagonizes apomorphine-induced turning in the rat model (Cowan, Dettmar & Walter, 1975b). These effects may be related to an ability of buprenorphine to release dopamine in the central nervous system but an indirect action on noradrenergic or tryptaminergic pathways cannot be excluded. To investigate these possibilities, the concentrations of homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5HIAA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) were simultaneously measured in the forebrains (containing frontal cortex, basal ganglia and hypothalamus) of male Sprague-Dawley albino rats (150–180 g; $n=5$–6) 1 h after injection of buprenorphine hydrochloride (0.1, 3 and 10 mg base/kg, s.c.), haloperidol (0.3, 1 and 3 mg/kg, i.p.), or vehicle (5 ml/kg).

5-Hydroxyindoleacetic acid was measured using the method of Curzon and Green (1970) and MHPG was estimated using a semi-micro method based on the gas-liquid chromatographic procedure of Walter & Eccleston (1973); HVA was estimated using gas liquid chromatography after forming the 4′-trifluoracetyl, trifluorethyl ester of HVA.

Statistically significant increases in the concentration of HVA were obtained after injection of buprenorphine (0.1, 3 and 10 mg/kg, $P<0.01$ by Student's $t$ test) or haloperidol (0.3, 1 and 3 mg/kg, $P<0.01$) however, the concentrations of 5-HIAA and MHPG were not significantly affected by either compound.

Naloxone hydrochloride (3 mg salt/kg, s.c. at −5 min) antagonized the increase in HVA caused by buprenorphine (0.1 mg/kg, $P<0.05$; 3 mg/kg, $P<0.01$) but had no significant effect on the increase in HVA produced by haloperidol (1 mg/kg). At this dose level, naloxone did not significantly alter the concentrations of HVA, 5-HIAA or MHPG. The contrasting results obtained with naloxone are similar to the previous findings (Cowan, Dettmar & Walter, 1975b) that naloxone (3 mg/kg) antagonizes the inhibitory effect of buprenorphine (0.1 mg/kg), but not that of haloperidol (1 mg/kg), on apomorphine-induced turning behaviour in rats.

The present data suggest that doses of buprenorphine which cause behavioural changes may increase the turnover of dopamine, but not that of noradrenaline or 5-hydroxytryptamine, in the forebrain of rats. An opiate link would appear to be involved in the mediation of this effect since the rise in HVA is naloxone-sensitive.

### References

COWAN, A., DETTMAR, P.W. & WALTER, D.S. (1975a). Analgesics and rotational behaviour in rats with unilateral substantia nigra lesions. Effects in the presence and absence of (+)-amphetamine. *Br. J. Pharmac.*, **55**, 316P.

COWAN, A., DETTMAR, P.W. & WALTER, D.S. (1975b). The effects of buprenorphine, morphine and pentazocine on turning behaviour and stereotypy induced by apomorphine in the rat. *J. Pharm. Pharmac.*, **27** (Suppl.), 15P.

CURZON, G. & GREEN, A.R. (1970). Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of rat brain. *Br. J. Pharmac.*, **39**, 653–655.

WALTER, D.S. & ECCLESTON, D. (1973). Increase of noradrenaline metabolism following electrical stimulation of the locus coeruleus in the rat. *J. Neurochem.*, **21**, 281–289.

---

## The stimulated release of dopamine-$\beta$-hydroxylase from intact organs of normo- and hypertensive rats

D.R. ALGATE & G.D.H. LEACH

*School of Pharmacology, University of Bradford*

Measurement of the circulating levels of dopamine-$\beta$-hydroxylase (DBH), the mixed function oxidase catalysing the hydroxylation of dopamine to noradrenaline in postganglionic monoaminergic neurones and the adrenal medulla, has been proposed as a useful index of sympathetic nervous activity (Axelrod, 1972). Its application as a means of studying sympathetic nervous involvement in hypertension has not been accepted by all workers (Geffen, Rush, Louis & Doyle, 1973; Horwitz, Alexander, Lovenberg & Keiser, 1973) since the enzyme levels measured in serum from man and animals cannot be consistently shown to correlate with blood pressure changes as a result of increased sympathetic activity. However, raised circulating levels of DBH have been observed in certain types of hypertension in man and animals (De Champlain, Farley, Cousineau & van Ameringen, 1976; Schanberg & Kirshner, 1976).

Circulating DBH is believed to be derived from monoaminergic nerve-endings of all sympathetically innervated tissues, therefore estimation of DBH in the circulation is not a satisfactory measurement of the stimulated release of neuronal DBH. An alternative is described below.

Experiments were performed on female rats (C.F.E. derived strain) weighing 200–240 grams. The animals were prepared for stimulation of the sympathetic outflow using a modification of the Gillespie and Muir preparation (1967) and artificially respired. The splenic vein of eight rats was cannulated and connected to a cannula in the left femoral vein to maintain haemodynamic continuity. The hepatic-portal vein of a further eight rats was cannulated and similarly connected to a femoral vein cannula. The blood pressure was monitored in all animals by means of a cannula placed in the left carotid artery. Blood samples were collected from the spleen or hepatic-portal vein following selective stimulation of the sympathetic outflow supplying the coeliac and superior mesenteric ganglia (T10-L1), at frequencies between 1 and 25 Hz (20 V, pulse-width 0.3 ms for 5 s). At the same time blood samples were taken from the carotid artery to serve as controls. DBH released from heart tissue was measured by sampling from the aortic arch of eight rats following selective stimulation at frequencies between 0.5 and 5 Hz (30 V, pulse-width 0.5 ms for 15 s) of the spinal outflow to the stellate ganglion (C7-T1). Serum was removed simultaneously from the femoral vein to serve as a control. These experiments were repeated using various types of hypertensive rats. The animal models used were spontaneously hypertensive, renal hypertensive and deoxycorticosterone acetate/NaCl rats.

The serum was assayed for DBH activity by the spectrophotometric method of Kato, Kuzuya & Nagatsu (1974).

In each of the three tissues, DBH was shown to be released in a frequency dependent manner. The maximum enzyme levels released in response to stimulation expressed as $\text{nmol.ml}^{-1}.\text{h}^{-1} \pm$ s.e. mean, were spleen, $25.25 \pm 2.25$, (25 Hz); mesentery, $14.3 \pm 0.95$, (25 Hz); and heart $9.1 \pm 1.7$ (5 Hz). The stimulated release of the enzyme in hypertensive animals did not differ significantly from those of the normotensive rats.

These results provide further evidence for the combined release of DBH and noradrenaline at nerve endings. They also suggest that biochemical mechanisms associated with the release of transmitter at the sympathetic postganglionic nerve-ending do not differ significantly in hypertensive rats.

### References

AXELROD, J. (1972). Dopamine-β-hydroxylase. Regulation of its synthesis and release from nerve terminals. *Pharmac. Rev.*, **24**, 233–243.

DE CHAMPLAIN, J., FARLEY, L., COUSINEAU, D. & VAN AMERINGEN, M. (1976). Circulating catecholamine levels in human and experimental hypertension. *Circ. Res.*, **38**, 109–114.

GEFFEN, L.B., RUSH, R.A., LOUIS, W.J. & DOYLE, A.E. (1973). Plasma dopamine-β-hydroxylase and noradrenaline amounts in essential hypertension. *Clin. Sci.*, **44**, 617–620.

GILLESPIE, J.S. & MUIR, T.C. (1967). A method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat. *Br. J. Pharmac. Chemother.*, **30**, 78–87.

HORWITZ, D., ALEXANDER, R.W., LOVENBURG, W. & KEISER, H.R. (1973). Human serum dopamine-β-hydroxylase. Relationship to hypertension and sympathetic activity. *Circ. Res.*, **32**, 594–599.

KATO, T., KUZUYA, H. & NAGATSU, T. (1972). A simple and sensitive assay for dopamine-β-hydroxylase activity by dual wavelength spectrophotometry. *Biochem. Med.*, **10**, 320–328.

SCHANBERG, S.M. & KIRSHNER, N. (1976). Commentary: Serum dopamine-β-hydroxylase as an indicator of sympathetic activity and primary hypertension. *Biochem. Pharmacol.*, **25**, 617–621.

---

## Evidence for tryptamine receptors on cardiac sympathetic nerves

J.R. FOZARD & A.T.M. MOBAROK ALI

*Department of Pharmacology, Materia Medica and Therapeutics, The University, Manchester M13 9 PT.*

5-Hydroxytryptamine (5-HT) stimulates noradrenaline release from rabbit cardiac sympathetic nerves (Fozard & Mwaluko, 1976). Despite the fact that classical tryptamine receptor antagonist drugs displayed no selective blocking activity, the possibility was considered that tryptamine receptors (Gaddum & Picarelli, 1957) exist on the terminal fibres which evoke transmitter release when activated (Fozard & Mwaluko, 1975). In an attempt to clarify the situation, the effects of several analogues of 5-HT have been investigated on the rabbit heart and comparisons have

**Table 1** Equivalent molar ratios for stimulation of the cardiac sympathetic nerves and the ileal cholinergic nerves

| | *Molar dose ratio* $\frac{ED_{50}\ drug}{ED_{50}\ 5\text{-}HT}$ | |
|---|---|---|
| Compound | *Heart* | *Ileum* |
| 5-HT | 1 | 1 |
| *N*-methyl-5-HT | 2.2 (1.7–2.8) (4) | 1.2 (0.3–4.5) (4) |
| *N,N*-dimethyl-5-HT | 2.3 (1.8–3.0) (5) | 2.9 (1.2–6.6) (7) |
| 5,6-dihydroxytryptamine | 4.7 (3.2–6.9) (3) | 3.5 (3.0–4.0) (4) |
| Tryptamine | 302* (135–676) (4) | 328* (133–807) (5) |

Figures presented are mean values with 95% confidence limits. * Molar dose ratio $\frac{ED_{25}\ \text{drug}}{ED_{25}\ \text{5-HT}}$

been made with their potency as stimulants of the cholinergic elements of the guinea pig ileum.

Hearts were perfused with modified Tyrode solution containing atropine (1.4 μM) and right atrial and ventricular tensions and cardiac rate were recorded as previously described (Fozard & Muscholl, 1971). Segments of guinea pig ileum were set up in modified Tyrode solution containing methysergide (2.8 μM).

Bolus injections of 5-HT, tryptamine, 5,6-dihydroxytryptamine, *N*-methyl-5-HT and *N,N*-dimethyl-5-HT increased the force and rate of cardiac contraction. With the exception of *N,N*-dimethyl-5-HT, the $ED_{50}$ responses of all the compounds were abolished by perfusion with 5-HT (7.1 μM), colchicine (1 mM) or propranolol (0.34 μM) indicating a basically similar mechanism of indirect sympathomimetic action. Responses to *N,N*-dimethyl-5-HT were abolished by colchicine or propranolol, but a small residual response occasionally remained during perfusion with 5-HT. All the compounds also stimulated the guinea pig ileum treated with methysergide. Responses to the $ED_{50}$ of all the compounds were abolished by morphine (1.3 μM) or atropine (1.4 μM) confirming activation of the cholinergic neuronal elements of the ileum. The equiactive molar ratios for stimulation of the cardiac sympathetic nerves and the ileal cholinergic nerves are presented in Table 1.

Thus, four closely related analogues of 5-HT stimulate transmitter release from rabbit cardiac sympathetic nerves by a mechanism basically similar to 5-HT itself. The close similarity between the potencies of these compounds relative to 5-HT in this respect and on the cholinergic nerves of the guinea pig ileum would implicate a common receptor site.

## References

FOZARD, J.R. & MUSCHOLL, E. (1971). A useful muscarinic parameter and the differential recording of atrial and ventricular tension in the perfused rabbit heart. *Naunyn-Schmiedeberg's Arch. Pharmak.*, **270**, 319–325.

FOZARD, J.R. & MWALUKO, G.M.P. (1975). The stimulant response of 5-hydroxytryptamine on cardiac sympathetic nerves: Resistance to blockade by classical tryptamine receptor antagonists. *Proceedings of the 6th International Congress of Pharmacology, July 20–25, 1975, Helsinki, Finland,* Abstract 697.

FOZARD, J.R. & MWALUKO, G.M.P. (1976). Mechanism of the indirect sympathomimetic effect of 5-hydroxytryptamine on the isolated heart of the rabbit. *Br. J. Pharmac.*, **57**, 115–125.

GADDUM, J.H. & PICARELLI, Z.P. (1957). Two kinds of tryptamine receptors. *Brit. J. Pharmacol.*, **12**, 323–328.

# Inhibitory effect of clonidine on a peripheral adrenergic synapse

O.A. IDOWU & M.A. ZAR

*Department of Pharmacological Sciences, The Medical School, The University of Newcastle upon Tyne, NE1 7RU.*

Clonidine is a potent hypotensive agent and is known to possess powerful central nervous system effects (Schmitt, Schmitt, Boissier & Giudicelli, 1967). Its peripheral actions have generally been regarded to be too insignificant to contribute to its hypotensive effect (Kobinger, 1975). Investigations of its action upon the release of noradrenaline (NA) in peripheral adrenergically innervated tissues have led to conflicting conclusions (Werner, Starke & Schümann, 1972; Stjärne, 1975). In the present study an attempt has been made to analyse the peripheral effects of clonidine at concentrations comparable to those observed in human plasma during antihypertensive therapy (Dollery, Davies, Draffan, Dargie, Dean, Reid, Clare & Murray, 1976) on a suitable adrenergically innervated *in vitro* preparation. The rat isolated anococcygeus (Gillespie, 1972) was used because of its ability to give reproducible mechanical responses to both long and short trains of electrical pulses.

Isolated anococcygii from male rats were set up in 10 ml organ baths between parallel platinum electrodes in Krebs–Henseleit at 37°C and contractions were recorded isometrically. For electrical field stimulation, pulses of 1 ms duration at 12 V were used.

Clonidine had a profound inhibitory effect on adrenergic motor transmission evoked by short trains ($<10$ pulses). Its effect declined as the train-length was increased (% inhibition at 10 Hz: $91.9 \pm 5.3$ with 2 pulses; $1.5 \pm 3.0$ with 40 pulses). Another factor which determined the intensity of inhibition was the frequency of pulses in each train; inhibition was more marked at lower frequencies (% inhibition of 5-pulse trains: $96.8 \pm 1.9$ at 1 Hz; $63.7 \pm 6.8$ at 40 Hz).

The threshold concentration of clonidine for inhibition was around $10^{-10}$M and maximum inhibition was achieved by concentrations of $10^{-9}$M–$10^{-8}$M.

NA-evoked contractions were not inhibited by clonidine, indicating that clonidine has a presynaptic site mediating its inhibitory effect on adrenergic transmission. Unlike clonidine, neither NA ($10^{-9}$ M – $10^{-6}$ M), nor phenylephrine ($10^{-9}$ M – $10^{-7}$ M), appeared to reduce the twitch-height. The indirect sympathomimetic tyramine ($10^{-7}$ M – $10^{-5}$ M) induced contraction of the smooth muscle, but did not inhibit the transmission. Inhibition due to clonidine was unaffected by the concurrent presence of tyramine ($10^{-5}$ M) or NA ($10^{-6}$ M) in the bath. The inhibitory effect of clonidine persisted unimpaired after blockade of neuronal uptake of NA by cocaine ($10^{-6}$ M) or by desmethylimipramine ($3 \times 10^{-8}$ M – $3 \times 10^{-7}$ M), and exogenous NA still lacked an inhibitory effect.

These results suggest that clonidine in low concentrations is an effective adrenergic neurone blocking agent; train-length and the frequency of stimulation are the critical factors, in that order, limiting its effectiveness. The mechanism of its inhibitory action involves presynaptic sites upon which NA, exogenous or endogenous, and phenylephrine have little or no effect.

We thank Boehringer, Ingelheim for the generous donation of clonidine hydrochloride.

## References

DOLLERY, C.T., DAVIES, D.S., DRAFFAN, G.H., DARGIE, H.J., DEAN, C.R., REID, J.L., CLARE, R.A. & MURRAY, S. (1976). Clinical pharmacology and pharmacokinetics of clonidine. *Clin. pharmacol. Ther.*, **19**, 11–17.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmac.*, **45**, 404–416.

KOBINGER, W. (1975). Central cardiovascular actions of clonidine. In: *Central action of drugs in blood pressure regulation*, eds. Davies, D.S. & Reid, J.L., pp. 181–193. Pitman Medical: Tunbridge Wells.

SCHMITT, H., SCHMITT, H., BOISSIER, J.R. & GIUDICELLI, J.F. (1967). Centrally mediated decrease in sympathetic tone induced by 2-(2,6-dichlorophenylamino)-2-imidazoline (St. 155, Catapressan). *Eur. J. Pharmac.*, **2**, 147–148.

STJÄRNE, L. (1975). Clonidine enhances the secretion of sympathetic neurotransmitter from isolated guinea-pig tissues. *Acta physiol. scand.*, **93**, 142–144.

WERNER, U., STARKE, K. & SCHÜMANN, H.J. (1972). Actions of clonidine and 2-(2-methyl-6-ethyl-cyclohexylamino)-2-oxazoline on postganglionic autonomic nerves. *Arch. int. Pharmacodyn.*, **195**, 282–290.

## Studies on monoamine oxidase in rat spinal cord

A.J. GEORGE & G.T. JONES

*Pharmacology Department, School of Pharmacy, Liverpool Polytechnic, Liverpool, UK.*

Monoamine oxidase (MAO) exists in at least two forms ('A' & 'B') which differ in their affinities for different amine substrates (Johnston, 1968). In this study, rat spinal cord and brain MAO activities were compared. Male rats (200–250 g) were killed and the spinal cord was removed, blotted, weighed, and then homogenized in ice-cold sucrose (0.25 M). Each homogenate was centrifuged at $8 \times 10^2$ for 5 min and the precipitate retained and resuspended in phosphate buffer (pH 7.2). Brain MAO was prepared as described by Johnston (1968). To assess MAO activity, 0.1 ml of suspension was incubated at 37°C for 1 h with 1 µ mol of [$^{14}$C]-tyramine (0.3 µ Ci/µmol) and phosphate buffer pH 7.2. A blank was prepared by boiling a 0.1 ml sample of the suspension. The reaction was terminated with HCl and [$^{14}$C]metabolite was extracted with ethyl acetate : benzene (1:1 v/v). 1 ml of extract was assayed for radio-activity (George & Leach, 1975) and the protein concentration of a 0.1 ml sample of suspension was determined as described by Lowry, Rosebrough, Farr & Randall (1951). The experiments were repeated using [$^{14}$C]-5-HT and [$^{14}$C]-benzylamine as substrates. The effects of the MAO inhibitors clorgyline and pargyline were investigated, with regard to enzyme and substrate specificity.

When tyramine is the substrate, clorgyline ($1 \times 10^{-7}$ M), a specific inhibitor of MAO 'A' (Johnston, 1968) causes a greater inhibition of MAO activity in spinal cord (80%) than in brain (41%). Pargyline ($1 \times 10^{-7}$ M), a specific MAO 'B' inhibitor (Squires, 1972), inhibited brain MAO activity by 63% and spinal cord MAO activity by 24% using tyramine as substrate. Since tyramine is a substrate for both MAO types, it appears that type A activity is proportionately greater in rat spinal cord than in rat brain.

*Mean MAO activity as n moles product formed per mg protein $h^{-1}$*

| | *5-HT* | | | *Tyramine* | | | *Benzylamine* | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Control* | *C* | *P* | *Control* | *C* | *P* | *Control* | *C* | *P* |
| Brain | 20.62 | 2.31 | 17.97 | 9.64 | 3.70 | 6.10 | 7.21 | 6.98 | 1.05 |
| | ±1.98 | ±0.24 | ±1.83 | ±0.89 | ±0.29 | ±0.58 | ±0.78 | ±0.67 | ±0.09 |
| Spinal cord | 16.51 | 1.62 | 14.96 | 7.56 | 1.40 | 5.92 | 3.27 | 3.21 | 0.25 |
| | ±1.67 | ±0.18 | ±1.32 | ±0.71 | ±0.13 | ±0.60 | ±0.36 | ±0.34 | ±0.23 |

*C*=clorgyline $1 \times 10^{-7}$ M, *P*=pargyline $1 \times 10^{-7}$ M
Each result is the mean of 7 experiments.

This work was supported by a grant from W. Warner and Co.

### References

GEORGE, A.J. & LEACH, G.D.H. (1975). The involvement of $Ca^{2+}$ and $Mg^{2+}$ in the spontaneous and drug induced release of $^3$H-noradrenaline from mesenteric arteries. *Biochem. Pharmac.*, **24**, 737–741.

JOHNSTON, J.P. (1968). Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem. Pharmac.*, **17**, 1285–1297.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.L. (1951). Protein measurement with the Folin phenol reagent*J. Biol. Chem.*, **193**, 265–275.

SQUIRES, R.F. (1972). Multiple forms of monamine oxidase in intact mitochronia. In: *Monoamine Oxidases—New Vistas Biochemical Psychopharmacology*, Vol. 5, eds. Costa, E. & Sandler, M., pp. 355–371. Raven Press: New York.

# Effect of denervation and cocaine on the response of isolated rat vas deferens to noradrenaline and methoxamine

A.R. DEHPOUR, F. GHAFFARPOUR, K. HOSSEINZADEH & M.A. KHOYI
(introduced by F. MICHAL)

*Department of Pharmacology, Faculty of Medicine, University of Tehran, Tehran, Iran*

In the rat vas deferens, cocaine increases the maximum response and shifts the dose-response curve for noradrenaline (NA) to the left (Barnett, Greenhouse & Taber, 1968). The former has been ascribed to a postjunctional effect and the latter to the prevention of neuronal NA uptake by cocaine (Trendelenburg, 1972). To provide evidence for the site of action we used methoxamine, an alpha-adrenoceptor agonist, whose neuronal uptake is negligible (Trendelenburg, Maxwell & Pluchino, 1970).

Vasa deferentia of rats were placed in oxygenated Tyrode solution at 37°C, and the isotonic contractions were recorded on a kymograph and developed tension on Grass polygraph. Denervation of tissue was carried out by desheathing (Birmingham, 1970) 24 h before experiment. Reserpinization was achieved by reserpine (5 mg/kg and 2.5 mg/kg, 48 and 24 h before experiment respectively).

Cocaine (2.94 μM), potentiated the effect of NA 12-fold and increased the maximum response by 75 ± 12.4%. Denervation potentiated the response to NA ten-fold. Cocaine had no significant effect on the dose-response curve and maximum response to NA in denervated vasa deferentia.

ED$_{50}$ of methoxamine was 1.49 ± 0.36 μM in intact tissue and 2.83 ± 0.81 μM in reserpinized tissue. Devervation did not alter ED$_{50}$ of methoxamine significantly (4.32 ± 0.77 μM in reserpinized tissue) and phentolamine shifted the dose-response curve to the right in a parallel fashion.

ED$_{50}$ for methoxamine after pretreatment with cocaine was 1.49 ± 0.40 μM in intact tissue and 4.36 ± 1.01 μM in reserpinized tissue. Cocaine increased the maximum response to methoxamine by 3.5 ± 5.3% and 13 ± 2.7% in intact and reserpinized tissue respectively. This was significantly lower than that observed for NA.

These results support the idea that cocaine potentiates the response to NA by inhibiting the neuronal uptake of NA. They also suggest that the increase in maximum response to NA in intact rat vasa deferentia is at least partly due to prejunctional effects of cocaine.

### References

BARNETT, A., GREENHOUSE, D.D. & TABER, R.I. (1968). A new type of drug enhancement: increased maximum response to cumulative noradrenaline in the isolated rat vas deferens. *Br. J. Pharmac.*, **33**, 171–176.

BIRMINGHAM, A.T. (1970). Sympathetic denervation of the smooth muscle of the vas deferens. *J. Physiol (Lond.)*, **206**, 645–661.

TRENDELENBURG, U. (1972). Factors influencing the concentration of catecholamines at the receptors. In *Handbook of Experimental Pharmacology*, Vol. **33**, eds. Blaschko, H. & Muscholl, E., pp. 726–761. Springer-Verlag: Berlin.

TRENDELENBURG, U., MAXWELL, R.A. & PLUCHINO, S. (1970). Methoxamine as a tool to assess the importance of intraneuronal uptake of 1-norepinephrine in the cat's nictitating membrane. *J. Pharmac. exp. Ther.*, **172**, 91–99.

---

# The effects of AH 5158 on metabolism of $^3$H(–)noradrenaline released from the cat spleen by nerve stimulation

R.J. SUMMERS & JANET TILLMAN

*Department of Pharmacology, The University, Glasgow G12 8QQ*

In the isolated blood perfused cat spleen AH 5158, 5-(1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl) salicylamide, acts as a selective post-synaptic α-adrenoceptor antagonist. The drug elevates transmitter overflow but the mechanism involves inhibition of uptake rather than inhibition of pre-synaptic α-adrenoceptors (Blakeley & Summers, 1976). The present experiments investigate whether the site of uptake inhibition is neuronal or extraneuronal and whether AH 5158 affects degradative enzymes.

Isolated blood perfused cat spleens were labelled with [$^3$H]-(–)-noradrenaline (0.5 nM/min for 10 min; blood flow 6.0 ± 0.3 ml/min, $n = 9$). Loosely bound label was washed out by perfusion with Krebs bicarbonate saline for 30 minutes. Blood perfusion was re-established and the spleen perfused for 30 minutes. After stimulation of the splenic nerves with 200 impulses at 10 Hz, venous blood was collected in four 1 min fractions, chilled and centrifuged. One aliquot of plasma was counted directly and another was taken for the separation of [$^3$H]-(–)-noradrenaline and its metabolites by column chromatography (Graefe, Stefano & Langer, 1973).

AH 5158 ($10^{-7}-3\times10^{-4}$ M) produced a dose dependent increase in the overflow of [$^3$H] following nerve stimulation ($r=0.83$; $P<0.02$; $n=8$). The dose required to produce a 50% increase in overflow was $2.3\times10^{-5}$M. These results are similar to those obtained by measurement of the overflow of endogenous noradrenaline (Blakeley & Summers, 1976).

AH 5158 had no apparent effect on the production of [$^3$H]-DOMA [3,4-dihydroxy mandelic acid] or the O-methylated deaminated metabolites, [$^3$H]-MOPEG [3-methoxy 4-hydroxy phenyl glycol and [$^3$H]-VMA [3-methoxy 4-hydroxy mandelic acid], indicating that the drug does not directly inhibit MAO, COMT or extraneuronal uptake. [$^3$H]-DOPEG (3:4 dihydroxy phenyl ethylene glycol) production was inhibited, particularly during the last 3 min of collection when, under normal conditions, a high proportion of [$^3$H] overflowed as metabolites. The inhibition was dose-dependent and the doses required to reduce [$^3$H]-DOPEG production by 50% in the last 3 min of collection were $1.2\times10^{-5}$ M, $2.3\times10^{-5}$ M and $2.4\times10^{-5}$ M. There was a significant ($r>0.885$, $P<0.001$, $n>6$) inverse relationship between the change in proportion of [$^3$H]-(–)-noradrenaline and [$^3$H]-DOPEG recovered for each of the last three 1 min collection periods. DOPEG is thought to be derived from the fraction of transmitter taken back into the nerves which is metabolized by MAO and aldehyde reductase (Kopin, 1972), and the amount formed has been taken as an index of neuronal uptake (Cubeddu, Barnes, Langer & Weiner, 1974). Since both the neuronal uptake inhibitor cocaine (Cubeddu *et al.*, 1974) and AH 5158 inhibit the production of [$^3$H]-DOPEG following nerve stimulation it is likely that the site of uptake inhibition by AH 5158 is neuronal.

This work was supported by the Wood Boyd Fund of Glasgow University. JT is an MRC Scholar.

### References

BLAKELEY, A.G.H. & SUMMERS, R.J. (1976). The effects of AH 5158 on the overflow of transmitter and the uptake of [$^3$H]-(–)-noradrenaline in the cat spleen. *Br. J. Pharmac.*, **56**, 364–365P.

CUBEDDU, L.X., BARNES, E., LANGER, S.Z. & WEINER, N. (1974). Release of norepinephrine and dopamine-$\beta$-hydroxylase by nerve stimulation, I. Role of neuronal and extraneuronal uptake and of alpha presynaptic receptors. *J. Pharmac. exp. Ther.*, **190**, 431–450.

GRAEFE, K.H., STEFANO, F.J. & LANGER, S.Z. (1973). Preferential metabolism of $^3$H-norepinephrine through the deaminated glycol in the cat vas deferens. *Biochem. Pharmac.*, **22**, 1147–1160.

KOPIN, I.J. (1972). Metabolic degradation of Catecholamines. The relative importance of different pathways under physiological conditions and after administration of drugs. In: *Handbook Exper. Pharm. XXXIII*—Catecholamines, eds. Blaschko, H. & Muscholl, E. Springer-Verlag: Berlin and Heidelberg, New York.

---

## The receptors involved in catecholamine mediated growth and secretion in rat parotid gland

A.D. CORBETT, BARBARA E. MAIN, T.C. MUIR & D. TEMPLETON

*Department of Pharmacology, The University, Glasgow G12 8QQ*

Catecholamines increase the rate of growth and produce a protein-rich secretion in rat salivary glands (Schneyer, 1974; Muir, Pollock & Turner, 1975). These effects are inhibited by propranolol and are presumably mediated via $\beta$-adrenoceptors. The increase in growth may arise from a depletion of secretory material (especially amylase) following catecholamine stimulation (Schneyer, 1974). This hypothesis was investigated by comparing the ability of sympathetic nerve stimulation and several adrenergic agonists to enhance growth and deplete amylase and total protein stores. A classification of the $\beta$-adrenoceptor involved in terms of $\beta_1$ or $\beta_2$ activity (Lands, Arnold, McAuliff, Luduena & Brown, 1967) was also made.

In anaesthetized (halothane 3%, $N_2O/O_2$ 2:1) rats the cervical sympathetic trunk or the parasympathetic nerves to the parotid were stimulated (20 Hz, 1 ms, 30 s/min supramaximal for up to 90 min) unilaterally or drugs given i.p. and saliva collected from the cannulated duct. Pieces of gland (10–20 mg) were removed before and again after (90 min) treatment and the total protein (Lowry, Rosebrough, Farr & Randall, 1951) and amylase levels (Phadebas commercial kit) compared. Saliva was also assayed for total protein and amylase content. Total protein and amylase content of unstimulated contralateral glands measured before and after nerve stimulation were not significantly different. Increased growth was measured, as an increased DNA synthesis by the uptake of [$^3$H]-thymidine and as an increase in the mitotic index determined 32 h after commencing treatment and 8 h after colchicine (1 mg/kg i.p.). Controls were contralateral unstimulated glands or glands from saline treated animals (Drug experiments).

Isoprenaline (100 n mol/g) and sympathetic nerve stimulation 90 min but not salbutamol (100 n mol/g) or oxymetazoline (50 n mol/g) significantly increased growth. Isoprenaline, salbutamol and both sympathetic and parasympathetic nerve stimulation for 90 min evoked secretion. Parasympathetic nerve stimulation produced the highest volume (190 ± 34 µl $n=3$), salbutamol the lowest (15 ± 1 µl $n=3$) over 90 minutes. Isoprenaline, sympathetic nerve stimulation and salbutamol, unlike parasympathetic nerve stimulation produced an amylase-rich secretion. Isoprenaline and sympathetic nerve stimulation, but neither salbutamol nor parasympathetic nerve stimulation depleted protein and amylase levels by approximately 80% and 50% respectively after 90 minutes.

Only treatments which activate $\beta_1$-adrenoceptors (isoprenaline and sympathetic nerve stimulation) enhanced growth. Salbutamol mainly a $\beta_2$ agonist, oxymetazoline, an $\alpha$ agonist and parasympathetic nerve stimulation were ineffective. A similar picture emerges when the ability to deplete protein and amylase is considered. These results suggest that, (a) the mechanism responsible for catecholamine induced growth operates via a $\beta_1$-adrenoceptor and (b) only those treatments depleting residual secretory material cause a significant increase in the rate of gland growth.

DT is an MRC scholar and ADC a Carnegie scholar. The support of the Wood Boyd Fund of Glasgow University is gratefully acknowledged.

**References**

LANDS, A.M., ARNOLD, A., McAULIFF, J.P., LUDUENA, F.P. & BROWN, T.G. (1967). Differentiation of receptor systems activated by sympathomimetic amines. *Nature (Lond.)*, **214**, 597–598.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol Chem.*, **193**, 265–275.

MUIR, T.C., POLLOCK, D. & TURNER, C.J. (1975). The effects of electrical stimulation of the autonomic nerves and of drugs on the size of salivary glands and their rate of cell division. *J. Pharmac. exp. Ther.*, **195**, 372–381.

SCHNEYER, C.A. (1974). Autonomic regulation of secretory activity and growth responses of rat parotid gland. In: *Secretory mechanisms of exocrine glands*. Alfred Benzon Symposium, eds. Thorn, N.A. & Petersen, O.H., pp. 42–67. Munksgaard: Copenhagen.

---

## Studies on the marked antihypertensive properties of indapamide (SE 1520) in rats and cats

L. FINCH & P.E. HICKS

*School of Studies in Pharmacology, University of Bradford, Bradford, BD7 1DP*

SE 1520 (Indapamide, N-3-sulphamoyl-4-chlorobenzamide)-2-methyl-indoline) has been reported to be active in the treatment of mild to moderate hypertension (Seedat & Reddy, 1974; Whately & Heraty, 1976). The mode of action of SE 1520 as an antihypertensive agent was, therefore, studied in experimental hypertensive cats and rats.

In conscious renal hypertensive cats (Finch, 1975), SE 1520 (2 x 10 mg orally) produced a moderate fall in blood pressure without any accompanying change in the resting heart rate. In conscious DOCA/NaCl hypertensive rats ($n=4$), SE 1520 (2 x 10 mg/kg orally) produced a marked fall in the mean blood pressure for a period of 24 h when measured from cannulae implanted directly in the aortic arch (Finch, Hersom & Hicks, 1975). SE 1520 (10 mg/kg orally for 10 days) and hydrochlorothiazide (5 mg/kg i.p. for 10 days) also produced a marked hypotensive effect in the DOCA/NaCl hypertensive rats, when measured by the tail/cuff method.

In pithed rat preparations ($n=8$) pretreatment with SE 1520 (10 x 10 mg/kg orally) markedly reduced the pressor responses to noradrenaline, tyramine and stimulation of the entire sympathetic outflow (Gillespie & Muir, 1967), whilst pretreatment with hydrochlorothiazide (10 x 5 mg/kg i.p.) did not alter the cardiovascular reactivity of the pithed rat preparation. However, in both the isolated portal vein preparation and the Krebs perfused mesenteric artery preparation from rats pretreated with SE 1520 (10 x 10 mg/kg orally) the responses to noradrenaline were similar to those obtained using untreated rats.

In conclusion, SE 1520 exerts an antihypertensive action in experimental hypertensive animals and after a 10 day pretreatment also markedly reduced the cardiovascular reactivity to various pressor agents. Since no changes in cardiovascular reactivity could be observed after similar pretreatment with hydrochlorothiazide, SE 1520 may have a novel mode of action as an antihypertensive agent.

SE 1520 (Natrilix®) was donated by Servier Laboratories, Greenford, Middlesex.

**References**

FINCH, L. (1975). The central hypotensive action of clonidine and Bay 1470 in cats and rats. *Clin. Sci. & Mol. Med.*, **48**, 273s–276s.

FINCH, L., HERSOM, A. & HICKS, P. (1975). Studies on the hypotensive action of α-methyldopamine. *Br. J. Pharmac.*, **54**, 445–451.

GILLESPIE, J.S. & MUIR, T.C. (1967). A method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat. *Br. J. Pharmac. Chemother.*, **30**, 78–87.

SEEDAT, Y.K. & REDDY, J. (1974). Clinical evaluation of SE 1520 in the treatment of hypertension. *Curr. Ther. Res.*, **16**, 275–280.

WHATELY, W.E. & HERATY, P. (1976). A general practice evaluation of indapamide in the management of hypertension. *Practitioner*. In Press.

---

## Effect of endogenous metabolites on the binding of *o*-methyl red to human serum albumin

C.J. BOWMER & W.E. LINDUP

*Department of Pharmacology & Therapeutics, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX*

The binding of several acidic drugs and dyes to the plasma proteins of patients with renal insufficiency is decreased (Reidenberg & Affrime, 1973), and the dye *o*-methyl red, for example, shows a typical decrease (Breyer & Radcliff, 1954; Campion, 1973). Campion (1973) investigated the effect of adding some hydrophilic metabolites known to accumulate in uraemia on the binding of *o*-methyl red to normal serum and found a slight but non-significant decrease in binding. However, Odar-Cederlöf (1975) found evidence that retained uraemic metabolites may be responsible for the inhibition of binding of warfarin. We have therefore used equilibrium dialysis to investigate the effect of some endogenous metabolites, which accumulate in uraemia, on the binding of *o*-methyl red to human serum albumin (HSA) *in vitro*.

Hydrophilic metabolites were added to the albumin-dye solution 20 min prior to dialysis and fatty acids were added by the method of Spector & Hoak (1969).

**Table 1** Effect of endogenous metabolites on the binding of *o*-methyl red to human serum albumin. Equilibrium dialysis at 37°C was carried out with *o*-methyl red ($1.33 \times 10^{-4}$ M) and HSA ($1.45 \times 10^{-4}$ M) in 0.1 M phosphate buffer pH 7.4

| *Metabolite* | *Total concentration (mM)* | *Unbound dye (D) (%)* | *Percentage increase in D relative to control* |
|---|---|---|---|
| Indoxyl sulphate | 0 | 15.2 (±1.2)† | |
| | 0.1 – 1.0 | *17.7 (±0.8)–31.5 (±1.5) | 16–107 |
| Uric acid | 0 | 15.8 (±0.2) | |
| | 0.18 – 0.54 | 16.1 (±0.5)–15.8 (±0.1) | — |
| Phenol | 0 | 15.6 (±0.3) | |
| | 0.053 – 0.27 | 15.6 (±0.3)–16.0 (±0.5) | — |
| Urea | 0 | 15.4 (±0.3) | |
| | 4.2 – 16.7 | 15.0 (±0.2)–15.6 (±0.5) | — |
| Creatinine | 0 | 15.0 (±0.4) | |
| | 0.18 – 1.1 | 14.9 (±0.7)–14.5 (±0.3) | — |
| Lauric acid | 0 | 15.6 (±0.5) | |
| | 0.12 – 0.64 | *18.6 (±0.4)–54.8 (±0.7) | 19–251 |
| Myristic acid | 0 | 15.4 (±0.6) | |
| | 0.07 – 0.39 | *16.0 (±0.3)–37.9 (±0.6) | 4–146 |

* $P < 0.005$

† Each result is the mean (± s.d.) of five or more experiments

The fatty acid content of the solution was determined by gas chromatography. Each metabolite was studied over a range of albumin : metabolite ratios and control dialysis experiments were included for each study. Unbound *o*-methyl red in the diffusate was determined spectrophotometrically.

Table 1 summarises some of the results and shows that only one of the hydrophilic metabolites, indoxyl sulphate, produced a significant increase in the percentage of unbound *o*-methyl red.

A further nine hydrophilic metabolites had no effect. However, lauric acid and myristic acid both caused a marked increase in unbound dye concentration (Table 1). Renal insufficiency is accompanied by disorders of blood lipids (Cramp, Moorhead & Wills, 1975) and so lipophilic metabolites such as the fatty acids may be implicated in the decreased drug binding which occurs in uraemia. Preliminary spectroscopic investigations indicate that fatty acids inhibit the binding of *o*-methyl red to HSA by a mechanism different from that of indoxyl sulphate.

C.J.B. is an M.R.C. Scholar.

**References**

BREYER, B. & RADCLIFF, F.J. (1954). The adsorptive capacity of serum proteins in renal insufficiency. *Aust. J. Exp. Biol. Med. Sci.*, **32**, 411–419.

CAMPION, D.S. (1973). Decreased drug binding by serum albumin during renal failure. *Toxic. appl. Pharmac.*, **25**, 391–397.

CRAMP, D.G., MOORHEAD, J.F. & WILLS, M.R. (1975). Disorders of blood-lipids in renal disease. *Lancet*, **i**, 672–673.

ODAR-CEDERLÖF, I. (1975). Studies on the plasma protein binding of drugs in patients with renal failure. Thesis Karolinska Institute, Stockholm, Sweden.

REIDENBERG, M.M. & AFFRIME, M. (1973). Influence of disease on binding of drugs to plasma proteins. *Ann. N.Y. Acad. Sci.*, **226**, 115–126.

SPECTOR, A.A. & HOAK, J.C. (1969). An improved method for the addition of long-chain free fatty acid to protein solutions. *Analyt. Biochem.*, **32**, 297–302.

---

## *In vivo* absorption studies with paraquat and diquat in the dog

P.N. BENNETT, D.S. DAVIES &
GABRIELLE M. HAWKESWORTH

*Department of Clinical Pharmacology, Royal Postgraduate Medical School, London.*

Paraquat (1,1′-dimethyl-4,4′-bipyridilium) and diquat (1,1′-ethylene-2,2′-bipyridilium) are non-selective herbicides with a similar mode of action (Calderbank, 1964). Paraquat produces lung damage in man and in several species of laboratory animals, probably due to an energy-dependent accumulation of paraquat by the lung (Rose, Smith & Wyatt, 1974), whereas diquat is not actively accumulated by lung slices and is less toxic to the lung *in vivo*.

After i.v. administration of tracer doses of [$^{14}$C]-paraquat (30–50 µg/kg) to Greyhound dogs the plasma concentration-time curve fitted a tri-exponential decline, defined by the equation:

$$C = A^{-\alpha t} + B^{-\beta t} + C^{-\lambda t}$$

and the kinetics could be characterized on the basis of a three-compartment open linear system. Assuming that elimination occurs only from compartment 1, A, B, B, C, $\alpha$, $\beta$, and can be used to calculate the rate of constants of transfer between compartments (Rescigno & Segre, 1966). Employing the equations of Loo & Riegelman (1968) these rate constants can be used to calculate the amount of paraquat absorbed at any given time.

In fasting dogs the absorption of low doses of paraquat, administered in 200 ml water, was rapid but incomplete, reaching a peak plasma concentration 75 min after dosing. Oral administration of 120 µg/kg resulted in 46–66% absorption of paraquat in 6 h, whereas at doses of 2 mg/kg and 5 mg/kg, 22–38% and 25–28% respectively were absorbed. 70–90% of the paraquat absorbed, caluclated according to the Loo Riegelman equations, was excreted in the urine in 6 h and is consistent with the proportion recovered in the urine following i.v. administration. Diquat (12 µg/kg) was only 10–20% absorbed in 6 h and did not produce an early plasma peak. Administration of propantheline (15 mg i.v.) 15 min before an oral dose of paraquat altered the plasma concentration-time curve markedly, delaying the peak concentration by 3 h to 6 h, indicating that the stomach is not a major site of paraquat absorption.

The dose dependent absorption of paraquat in the dog suggests facilitated absorption, probably in the small intestine, whereas the absorption of diquat may occur solely by passive diffusion.

**References**

CALDERBANK, A. (1964). Mode of action of the bipyridilium herbicides paraquat and diquat. *Proc. 7th Brit. Weed Control Conference*, **1**, 312–320.

LOO, J.C.K. & RIEGELMAN, S. (1968). New method for calculating the intrinsic absorption rate of drugs. *J. Pharm. Sci.*, **57**, 918–928.

RESCIGNO, A. & SERGE, G. (1966). In: *Drug and Tracer Kinetics*. pp. 94–95. Blaisdell. New York.

ROSE, M.S., SMITH L.T. & WYATT, I. (1974). Evidence for energy-dependent accumulation of paraquat into rat lung. *Nature*, **252**, 314–315.

# Change in sensitivity to halothane in the rat following administration of sodium barbitone in the drinking water

M.J. TURNBULL[1] & J.W. WATKINS

*Department of Pharmacology, University of Dundee, Dundee, Scotland.*

We have measured the duration of halothane-induced anaesthesia at intervals during a four week period of administration of sodium barbitone and also after its subsequent withdrawal using the method we have previously described (Turnbull & Watkins, 1975a). Groups of eight female Wistar rats, weighing approximately 130 g at the beginning of the experiment, were used and the sodium barbitone was administered in increasing concentration in the drinking water (which also contained saccharin 125 mg/l) over the period of four weeks. The concentration of barbitone was such that the daily dose increased from approximately 100 mg/kg during the first week to approximately 400 mg/kg during the fourth week. The intended and actual barbitone intakes are compared in Figure 1. Drug withdrawal was effected by substituting saccharin solution for barbitone solution at the end of the fourth week. Control rats received saccharin solution throughout. Halothane-induced sleeping time was measured in treated and control rats at 2–3 day intervals during the period of drug administration and at more frequent intervals after drug withdrawal.

Compared with control animals, the sensitivity to halothane was found to decrease during each of the first three weeks of barbitone administration (Figure 1). Thus, the sleeping time was significantly prolonged when measured on the first occasion each week (days 2, 8 and 15) but by the end of each week (days 7, 14 and 21) the difference between treated and control groups was no longer statistically significant. However, during the fourth week, no such decrease in sensitivity was found, perhaps due to drug cumulation.

Following barbitone withdrawal, a significant decrease in sensitivity to halothane, indicative of an increased excitability of the central nervous system, rapidly developed and lasted for approximately 72 hours. This was followed by a period of hypersensitivity to halothane, but by the sixth day of withdrawal the sensitivity to halothane had returned to control values.

Thus the pattern of change in sensitivity to halothane following drug withdrawal after a four week period of barbitone administration is remarkably similar to that which we have reported to occcur following repeated injections of pentobarbitone for only 10 h (Turnbull & Watkins, 1975b). This suggests that the results obtained with our acute (10 h) experimental model may be predictive of the changes in central nervous system excitability likely to occur on chronic drug administration.

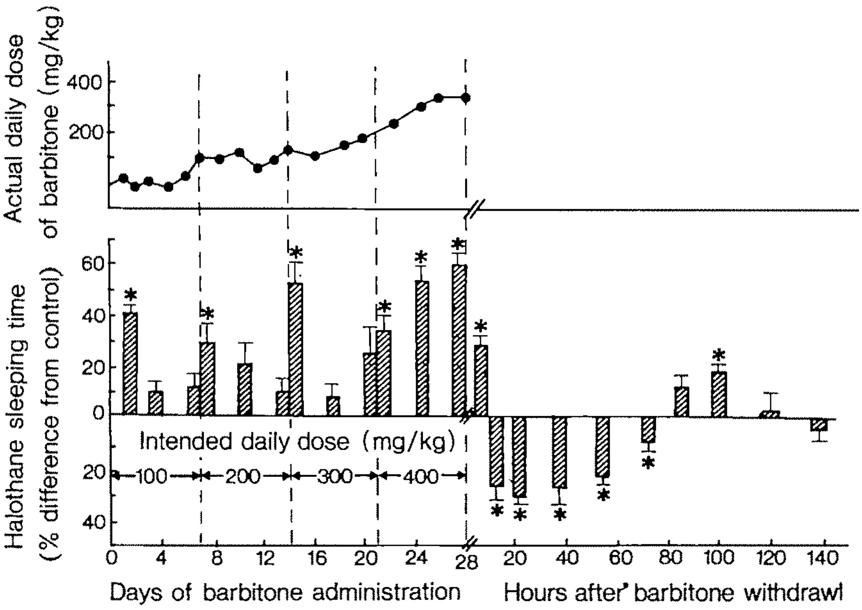


**Figure 1** Effect of chronic barbitone administration and withdrawal on the sensitivity to halothane in the rat.
*$P<0.05$

---

[1] Present address: I.C.I. Ltd., Alderley Park, Macclesfield.

The authors wish to thank the M.R.C. for financial assistance.

**References**

TURNBULL, M.J. & WATKINS, J.W. (1975a). The use of halothane induced sleeping time as an index of central nervous system excitability. *Br. J. Pharmac.*, 55, 307P.

TURNBULL, M.J. & WATKINS, J.W. (1975b). Further observations on the change in sensitivity to halothane induced by acute administration of central nervous sytem depressant drugs in the rat. *Br. J. Pharmac.*, 55, 266P.

---

# Effect of some receptor antagonists on fenfluramine-induced glucose uptake into the isolated rat hemidiaphragm

MARILYN J. KIRBY & P. TURNER

*Department of Clinical Pharmacology, St. Bartholomew's Hospital Medical College, London, EC1A 7BE.*

We have shown (Kirby, 1974; Kirby & Turner, 1975a,b) that the antiobesity drug fenfluramine in therapeutic concentrations causes a significant and dose related increase in glucose uptake into isolated rat and human skeletal muscle in the presence of insulin.

Using the rat hemidiaphragm preparation and fenfluramine (100 ng/ml), we have investigated the effect of a series of receptor blocking drugs (atropine, haloperidol, mepyramine, methysergide, propranolol and thymoxamine) on this phenomenon. The results (Table 1) show that only the 5-HT antagonist, methysergide, blocked the action of fenfluramine and that this effect was dose related occurring with relatively low concentrations. 10 ng/ml caused approximately 40% inhibition, the maximal response from earlier work being + 2.4 ± 0.60 mg of glucose/g wet weight of tissue in 90 min (Kirby & Turner, 1975b).

The results are in agreement with earlier work, in which methysergide was shown to block fenfluramine-induced contractions of human isolated saphenous vein (Kirby & Turner, 1971) and also with the evidence that the central effects of fenfluramine are mediated via 5-HT mechanisms (Garattini, Bizzi, de Gaetano, Jori & Samanin, 1975).

**Table 1** Effect of antagonist on fenfluramine-induced glucose uptake into the rat hemidiaphragm

| | | *Change with antagonist when compared with:* | |
|---|---|---|---|
| | | *(a) Insulin** | *(b) Insulin + fenfluramine** |
| *Antagonist* | *Concentration (ng/ml)* | *Response* | *Response* |
| Methysergide | 250 | −0.18 ± 0.46 | −1.90 ± 0.19** |
| | 50 | — | −1.98 ± 0.45* |
| | 10 | — | −0.87 ± 0.13* |
| | 2 | — | 0.15 ± 0.12 |
| Atropine | 250 | −0.13 ± 0.46 | −0.05 ± 0.21 |
| Haloperidol | 250 | −0.77 ± 0.45 | −0.17 ± 0.17 |
| Mepyramine | 250 | +0.12 ± 0.28 | +0.12 ± 0.17 |
| Propranolol | 250 | −0.58 ± 0.46 | −0.18 ± 0.34 |
| Thymoxamine | 250 | −0.18 ± 0.24 | +0.30 ± 0.50 |

* Change expressed as mg of glucose taken up/g wet weight of tissue in 90 min ± s.e. mean, insulin concentration 100 μu/ml, fenfluramine concentration 100 ng/ml, $n=6$ for all groups.
*$P<0.01$; **$P<0.001$ using paired $t$-test.

M.J.K. is a recipient of the Williams Fellowship for Medical and Scientific Research, London University.

**References**

GARATTINI, S., BIZZI, A., de GAETANO, G., JORI, A. & SAMANIN, R. (1975). Recent advances in the pharmacology of anorectic agents: Recent Advances in *Obesity Research*, 1, 354–367.

KIRBY, M.J. (1974). Dose related effects of fenfluramine and norafenfluramine on glucose uptake into isolated human skeletal muscle. *Br. J. clin. Pharmac.*, 1, 511–512.

KIRBY, M.J. & TURNER, P. (1971). Action of methysergide on fenfluramine-induced contractions of saphenous vein. *J. Pharm. Pharmac.*, 23, 801–802.

KIRBY, M.J. & TURNER, P. (1974). Effect of fenfluramine and norfenfluramine on glucose uptake by the isolated rat diaphragm. *Br. J. Pharmac.*, 50, 477P.

KIRBY, M.J. & TURNER, P. (1975a). Fenfluramine and norfenfluramine on glucose uptake into skeletal muscle. *Postgrad. med. J.*, 51 (suppl.) 73–76.

KIRBY, M.J. & TURNER, P. (1975b). Amphetamine and fenfluramine on glucose uptake into rat and human skeletal muscle. *Recent Advances in Obesity Research*, 1, 378–380.

---

# Some effects of D600, nifedipine and sodium nitroprusside on electrical and mechanical activity in rat portal vein

M. JETLEY & A.H. WESTON

*Department of Pharmacology Materia Medica and Therapeutics, The Medical School, Manchester, M13 9PT.*

In rat portal vein it has been proposed that contraction is associated with the release of superficially-bound $Ca^{2+}$ and that this release is triggered by extracellular $Ca^{2+}$ (Sigurdsson, Uvelius & Johansson, 1975). In the present experiments the effects of the so called calcium antagonist D600 [methoxyverapamil; 5-methyl-4-cyan-4-(3,4,5-trimethoxyphenyl)1-*N*-methyl-*N*-*β*-3,4-dimethoxy-phenylethyl)-aminohexane hydrochloride; Knoll], nifedipine [4-(2′-nitrophenyl)-2,-6-dimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine; BAY a 1040, Bayer] and sodium nitroprusside on the electrical and mechanical activity of rat portal vein have been examined.

In normal physiological salt solution (PSS, containing 25 mM bicarbonate buffer, bubbled with $95\%O_2/5\%CO_2$) both D600 (0.01–1 μM) and nifedipine (0.001–0.1 μM) shifted the noradrenaline dose-response curve to the right with a reduction in the maximum response. Sodium nitroprusside (0.1–10 μM) had no significant effect. Using a modified PSS (containing 10 mM MOPS [3-(*N*-morpholino) propanesulphonic acid; Calbiochem] buffer, bubbled with 100% $O_2$), the inhibitory effects of both D600 and nifedipine were antagonized by increasing the calcium concentration in the PSS (up to 80 mM).

The effects of the calcium antagonists on the mechanical and extracellularly-recorded electrical activity evoked by noradrenaline (1 μM; approximately an $ED_{80}$) were studied using a perfused capillary similar to that described by Golenhofen & v. Loh (1970). A Grass polygraph was used and the electrical and mechanical records were mathematically integrated to provide a quantitative measurement of drug responses. Sodium nitroprusside (0.1–10 μM) had no significant effect. D600 (0.01–1 μM) and nifedipine (0.001–0.1 μM) both reduced the mechanical activity evoked by noradrenaline (1 μM) to the same extent as observed in the tissue bath experiments. However, neither agent produced a reduction in electrical activity comparable with this reduction in mechanical activity. The degree of this electro-mechanical uncoupling was greater in the presence of nifedipine than in the presence of D600. When the electrical and mechanical responses produced by noradrenaline (1 μM) were examined in the presence of phentolamine (0.01–0.32 μM), both were similarly reduced. Higher concentrations of D600 (10 μM) and of nifedipine (1 μM) produced greater inhibition of spontaneous and noradrenaline-evoked electrical activity.

These results suggest that low-moderate concentrations of D600 and nifedipine prevent extracellular $Ca^{2+}$ from triggering the release of $Ca^{2+}$ from superficially-bound calcium stores. Higher concentrations, which reduce electrical activity to a greater extent are also able to antagonize transmembrane calcium flux. The inability of sodium nitroprusside to antagonize the phasic mechanical activity in rat portal vein is consistent with the work of Kreye, Baron, Lüth & Schmidt-Gayk (1975). These workers showed that sodium nitroprusside was most effective in antagonizing tonic mechanical responses in tissues where contraction was associated with a pool of calcium relatively independent of extracellular $Ca^{2+}$.

Generous gifts of D600 (Knoll) and nifedipine (Bayer) are gratefully acknowledged.

**References**

GOLENHOFEN, K. & v. LOH, D. (1970). Elektrophysiologische Untersuchungen zur normalen Spontanaktivität der isolierten taenia coli des Meerschweinchens. *Pflügers Arch.*, **314**, 312–328.

KREYE, V.A.W., BARON, G.D., LÜTH, J.B. & SCHMIDT-GAYK, H. (1975). Mode of action of sodium nitropresside on vascular smooth muscle. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **288**, 381–402.

SIGURDSSON, S.B., UVELIUS, B. & JOHANSSON, B. (1975). Relative contribution of superficially bound and extracellular calcium to activation of contraction in isolated rat portal vein. *Acta physiol. scand.*, **95**, 263–269.

---

# A simple *in vitro* preparation of mammalian tissue exhibiting properties of slow tonic skeletal muscle

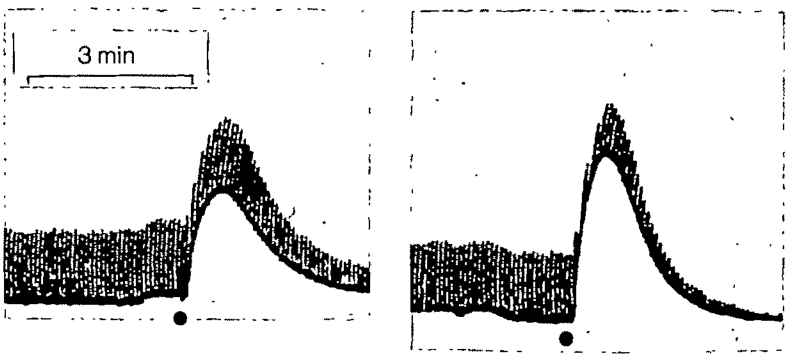
M. MAUREEN DALE &
R. MUID

*Department of Pharmacology, University College, London, Gower Street, London, WC1E 6BT.*

Guinea-pig cremaster muscle can be set up as an isolated preparation (Ninomiya, 1975; Dale, Evinc & Vine, 1976). It gives dose-related contractures with acetylcholine which, on the basis of the affinity constants obtained with (+)-tubocurarine and atropine, involve nicotinic not muscarinic receptors (Dale *et al.*, 1976). Set up as a simple nerve-muscle preparation, with isometric recording, the muscle manifested twitches to single supra-maximal stimuli. Repetitive stimulation at 20 Hz resulted in fused tetanus, but maximum tension (40 G) only developed above 60 Hz. The ratio of twitch tension to maximum tetanic tension was approximately 0.1. The muscle was singularly resistant to fatigue, tetanic contraction at 20 Hz being maintained up to 10 minutes.

KCl (0.1 M) produced a sustained increase in tension, and depolarizing neuromuscular blocking agents ($10^{-6}$—$5 \times 10^{-5}$ M) evoked slow, dose-related contractures, up to 70% of the maximum response to KCl. When added during repetitive nerve stimulation, succinylcholine, at low concentrations, produced a sustained increase in tension but, initially, no inhibition of twitch; total neuromuscular block involving both twitch and tension increase, occurred at $4 \times 10^{-5}$ M. (Figure 1).

Tetrodotoxin ($10^{-7}$ M) modified the dose-response curve to acetylcholine and succinylcholine, but substantial responses to these agents could still be obtained. On the nerve-muscle preparation, tetrodotoxin eliminated the twitch leaving the tension increase virtually unaltered. With tubocurarine it could be shown that these tetrodotoxin-resistant responses involved nicotinic not muscarinic receptors. It thus appears that the tissue contains skeletal muscle



a $1.6 \times 10^{-5}$M    b $4 \times 10^{-5}$m

**Figure 1** The response of the guinea-pig cremaster nerve/muscle preparation to succinylcholine. Bathing fluid: oxygenated Krebs-Henseleit solution at 32°C. Isometric recording with a Pye-Ether UFI transducer and George Washington Oscillograph 400 MD/2. Stimulation, using a Grass SD5 stimulator: 4 V, 0.2 Hz, 0.1 ms pulse duration.

fibres which do not require a propagated action potential for contraction.

These responses resemble those of mammalian extra-ocular muscles (Bach-y-Rita & Ito, 1966; Browne, 1976) and show some similarity to frog rectus and to avian muscle (Ginsborg, 1960) – all of which contain slow tonic skeletal muscle.

Preliminary histochemical studies of cholinesterase in cremaster muscle suggest that some fibres have innervation different from the typical 'en plaque' end-plates found in focally-innervated muscle.

Rigorous proof of the existence of a type of slow tonic muscle in the cremaster requires electrophysiological and electronmicroscopic studies. But the responses described above suggest fairly strongly that fibres of this type are present.

**References**

BACH-Y-RITA, P. & ITO, F. (1966). In vivo studies on fast and slow muscle fibres in cat extraocular muscles. *J. Gen. Physiol.*, **49**, 1177–1195.

BROWNE, J.S. (1976). The contractile properties of slow muscle fibres in sheep extraocular muscle. *J. Physiol. Lond.*, **254**, 535–550.

DALE, M.M., EVINÇ, A. & VINE, P. (1976). 'The Denervated cremaster muscle of the guinea-pig as a pharmacological preparation'. *Br. J. Pharmac.* (in press).

GINSBORG, B.L. (1960). Some properties of avian skeletal muscle fibres with multiple neuromuscular junctions. *J. Physiol. (Lond.)*, **154**, 581–598.

NINOMIYA, J.G. (1975). Some pharmacological properties of the cremaster muscle of the guinea-pig. *Br. J. Pharmac.*, **55**, 487–495.

---

## Fitting a general sigmoid model to pharmacological response curves

A. DAVEY, A. MARRIOTT &
M.A. STOCKHAM

*Allen and Hanburys Research Limited, Ware, Hertfordshire.*

In order to evaluate potency and drug activity it is necessary to quantify the relationship between the response and the dose. However, in some sets of data problems arise in a log-dose transformation and some of the data may have to be omitted.

Many attempts have been made to derive models from pharmacological principals (Ariens, 1954; Clark, 1937; Paton, 1961; Stephenson, 1956) but the most successful involve a function that can only be determined empirically, i.e. the efficacy.

Parker & Waud (1971) used the logistic curve

$$Y = \frac{1}{1 + A\exp(-bX)},$$

showing that it could be rearranged to give:

$$R(X) = M\frac{X^B}{X^B + C^B} \qquad (1)$$

Equation (1) has some of the required characteristics

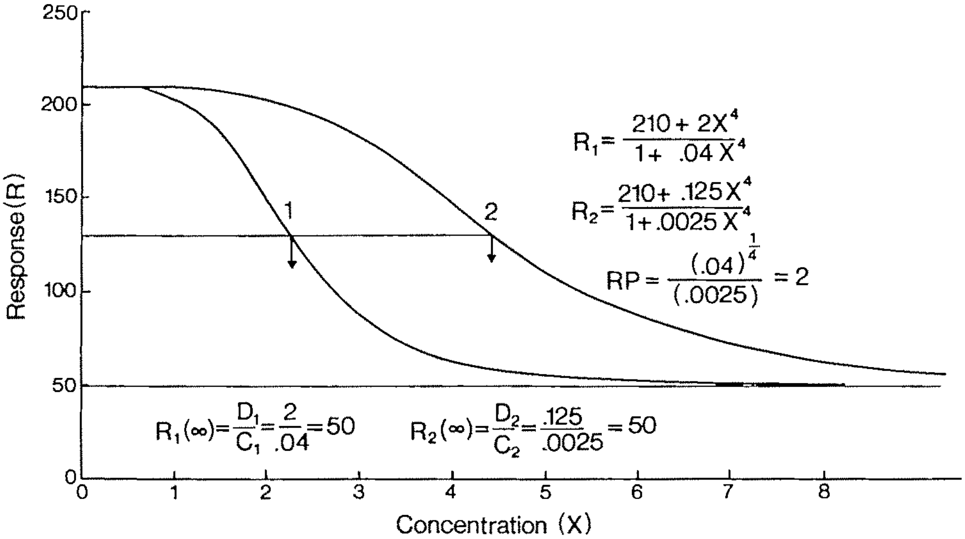


**Figure 1** Parallel sigmoid curves.

and potentially a number of pharmacological parameters could be represented.

We have modified the logistic curve to a more general form in order to define the parameters in terms of a number of useful practical pharmacological entities:

$$R(X) = \frac{A + DX^B}{1 + CX^B} \qquad (2)$$

This curve has the right characteristics, namely sigmoid shape. Two theoretical dose-response curves are shown in Figure 1 for drugs working by a similar mechanism but of different potency.

For the two response curves to be parallel they must both have the same asymptotic value and the same value of B. The asymptotic value of R(X) indicates the maximum achievable response and is where

$$R(X) = R(\infty) = \frac{D}{C}.$$

The control response is where $R(X) = R(O) = A$.

$$\left(\frac{1}{C}\right)^{1/B}$$

is the $ED_{50}$ and if B is the same value for the two curves the relative potency RB is

$$\left(\frac{C_1}{C_2}\right)^{1/B}.$$

Examples are shown for data having a Poisson distribution (from the acetylcholine writhing test), to data having a negative-exponential distribution (from the Roto-rod test), and for estimating potency of inhibitors of histamine release where the data was intractable by ordinary methods.

#### References

ARIËNS, E.J. (1954) Affinity and intrinsic activity in of competitive inhibition: problems and theory. *Arch. int. Pharmacodyn.*, 99, 32–49.

CLARK, A.J. (1937). The mode of action of drugs on cells. Baltimore: Williams & Wilkins.

PARKER, R.B. & WAUD, D.R. (1971). Pharmacological estimation of drug receptor dissociation constants. Statistical evaluation 1. Agonists. *J. Pharmac. exp. Ther.*, **117**, 1–12.

PATON, W.D.M. (1961). A theory of drug action based on the rate of drug receptor combination. *Proc. Roy. Soc. B.*, **154**, 21–69.

STEPHENSON, R.P. (1956). A modification of receptor theory. *Br. J. Pharmacol.*, **11**, 379–393.

---

## A postsynaptic action of prostaglandin $E_1$ on sympathetic responses in guinea-pig ileum

G.J. SANGER & A.J. WATT

*Department of Physiology, University of Manchester*

Prostaglandins inhibit the response of many tissues to sympathetic stimulation. It is now well established that one possible mechanism involved is a reduction of transmitter release (Hedqvist, 1969), but a postsynaptic action may sometimes also play a part (Clegg, 1966).

We have investigated the actions of prostaglandin $E_1$ on the responses of isolated guinea-pig ileum to stimulation of the perivascular nerves. The ileum was stimulated transmurally with just maximal stimuli at a frequency of 0.1 Hz. The perivascular nerve was stimulated with 8 s trains at a frequency of 1–32 Hz, immediately before each coaxial pulse (Watt, 1971). The frequency of perivascular stimulation required to produce a 50% inhibition of the coaxial twitch was increased by a factor of $1.5 \pm 0.15$ (mean ± s.e. mean $n = 10$ $P < 0.005$) in the presence of prostaglandin $E_1$ (57 nM). In similar experiments in which the coaxial twitch was inhibited by exogenous noradrenaline, prostaglandin $E_1$ (28 nM) increased the dose of noradrenaline necessary to produce a 50% inhibition by a factor of $2.3 \pm 0.4$; $n = 8$ ($P < 0.003$).

The effects of perivascular stimulation and of noradrenaline were also compared before and after treatment with indomethacin (7 µM). The frequency of perivascular stimulation then required to inhibit the coaxial twitch by 50% was $0.5 \pm 0.2$ of the frequency required to produce an equal inhibition of the untreated preparation. This effect of indomethacin was significant ($P < 0.015$).

Indomethacin also significantly reduced the concentration of noradrenaline necessary to inhibit the coaxial twitch by 50%; the concentration required being $0.3 \pm 0.1$ as great as that required in the untreated preparation ($P < 0.0002$). The results obtained with noradrenaline indicate that in the isolated guinea-pig ileum prostaglandin $E_1$, as well as any presynaptic action, has a postsynaptic action. The relatively greater effects both of prostaglandin $E_1$ and of indomethacin on the responses to noradrenaline than on the responses to perivascular stimulation, suggest an action of prostaglandin on adrenoreceptors which are not accessible to neurally released noradrenaline. One site for this action may be the β-adrenoceptors situated on the longitudinal muscle (Kosterlitz & Watt, 1965). Most of the sympathetic fibres terminate in Auerbach's plexus (Norberg, 1964) and the response to perivascular stimulation is hardly affected by β-adrenoceptor antagonists (Watt, 1971).

Possible explanations for the greater part of the postsynaptic action of prostaglandin are potentiation of the response to acetylcholine (Harry, 1968, Kadlec, Masek & Seferna, 1974) or an increase in the acetylcholine released from the Auerbach's plexus. However, in our experiments the response of guinea-pig ileum to acetylcholine was not affected by indomethacin (7 μM) confirming the findings of Bennett, Eley & Stockley (1975) and indicating that the effects observed in the presence of indomethacin were not due to inhibition of the response to acetylcholine.

G.J.S. is a Science Research Council scholar.

**References**

BENNETT, A., ELEY, K.G. & STOCKLEY, H.L. (1975). Modulation by prostaglandins of contractions in the guinea-pig ileum. *Prostaglandins*, **9**, 377–384.

CLEGG, P.C. (1966). Antagonism by prostaglandins of the responses to various smooth muscle preparations to sympathomimetics. *Nature (Lond.)*, **209**, 1137–1139.

HARRY, J.D. (1968). The action of prostaglandin-$E_1$ on the guinea-pig isolated intestine. *Br. J. Pharmac.*, **33**, 213P.

HEDQVIST, P. (1969). Modulating effect of prostaglandin-$E_2$ on noradrenaline release from the isolated cat spleen. *Acta. physiol. scand.*, **75**, 511–512.

KADLEC, O., MASEK, K. & SEFERNA, I. (1974). A modulating role of prostaglandins in contractions of the guinea-pig ileum. *Br. J. Pharmac.*, **51**, 565–570.

KOSTERLITZ, H.W. & WATT, A.J. (1965). Adrenergic receptors in the guinea-pig ileum. *J. Physiol. (Lond.)*, **177**, 11P.

NORBERG, K.A. (1964). Adrenergic innervation of the intestinal wall studied by fluorescence microscopy. *Int. J. Neuropharmacol.*, **3**, 379–382.

WATT, A.J. (1971). The inhibitory effect of perivascular stimulation on the responses of guinea-pig isolated ileum. *J. Physiol.*, **219**, 38–39P.

---

## The effect of subcutaneous injections of adrenaline on platelet MAO activity

R.C. BOURNE, C.D. FRITH, F. OWEN & G.J. RILEY (introduced by T.J. CROW)

*Division of Psychiatry, The Clinical Research Centre, Harrow.*

Gentil, Greenwood & Lader (1975) have recently reported large increases in platelet monoamine oxidase (MAO) activity following the subcutaneous injection of adrenaline and suggested that "stress" might produce rapid changes in the activity of the enzyme. In view of the relevance of this finding to the conflicting reports of a reduction of platelet MAO activity in schizophrenia (Murphy & Wyatt, 1972; Meltzer & Štahl, 1974; Shaskan & Becker, 1975; Bailey, Crow, Johnstone & Owen, 1975) we have attempted to verify the observations of Gentil and his colleagues and to improve the experimental design by (a) more frequent blood samples using an in-dwelling

**Table 1** The effect of adrenaline and placebo on platelet MAO activity

| *Benzylamine concentration* | *Sample* | *Injection received* | | * |
|---|---|---|---|---|
| | | *Placebo (n = 6)* | *Adrenaline (n = 6)* | t |
| 1 mM | Pre-injection | 27.8 ± 2.7 | 33.2 ± 6.3 | — |
| 1 mM | +20 min | 27.7 ± 3.9 | 37.3 ± 5.9 | 4.61† |
| 1 mM | +40 min | 31.0 ± 4.8 | 33.5 ± 5.6 | 0.32 |
| 1 mM | +60 min | 29.4 ± 4.3 | 33.7 ± 6.4 | 0.21 |
| 1 mM | +80 min | 29.8 ± 4.3 | 35.1 ± 6.6 | 0.11 |
| $2.1 \times 10^{-5}$ M | Pre-injection | 1.70 ± 0.30 | 1.82 ± 0.44 | — |
| $2.1 \times 10^{-5}$ M | +20 min | 1.82 ± 0.26 | 2.08 ± 0.37 | 1.93 |
| | Age (yrs) | 28.2 ± 5.6 | 27.5 ± 3.0 | 0.25 |

* '*t*' values refer to a comparison between pre- and post-injection samples of subjects receiving adrenaline only. † $P < 0.05$.
Results, mean ± s.d. expressed as nmoles product formed per mg protein 30 min$^{-1}$.

catheter, (b) determination of platelet counts on all samples and (c) the inclusion of a group receiving placebo.

Twelve male volunteers received subcutaneous injections of either water or adrenaline tartrate (1 : 1000 solution, 1 ml/70 kg body weight). Blood samples (20 ml) were collected into a citric acid-sodium citrate-dextrose buffer (ACD; 5 ml) for platelet MAO assay and 5 ml into disodium ethylenediamine tetraacetate (EDTA) for platelet count determination. Samples were taken, from an indwelling catheter in the ante-cubital vein, immediately prior to injection and at 20, 40, 60 and 80 min post-injection. Platelet MAO activity was assayed by the method of Robinson, Lovenberg, Keiser & Sjoerdsma (1968) with benzylamine as substrate at a concentration of 1 M for all samples, and at the much lower concentration of $2.1 \times 10^{-5}$M used by Gentil *et al.* (1975) for the first two samples only. Platelet counts were determined with a Technicon Autocounter. The results of the platelet MAO assays are presented in Table 1.

There was a small but significant increase ($P < 0.05$, paired '*t*' test) in platelet MAO activity of the group receiving adrenaline in the 20 min post-injection samples only, with benzylamine as substrate at 1 mM concentration. There was a small but not significant increase in the activity of platelet MAO, in both groups of subjects, with benzylamine at a concentration of $2.1 \times 10^{-5}$ M. Platelet counts were significantly increased in the 20 min ($P < 0.001$) 40 min ($P < 0.05$) and 60 min ($P < 0.05$) only in the group receiving adrenaline. As suggested by Gentil *et al.* (1975) the efflux of a different population of platelets from the spleen may account for the increase in platelet MAO activity observed in the 20 min post-injection samples.

We were unable, therefore, to fully confirm the findings of Gentil and his co-workers. The small changes in platelet MAO activity attributable to 'stress', at least in as far as it is mimicked by injections of adrenaline seem unlikely to have an important bearing on the current controversy over the activity of the enzyme in schizophrenia.

### References

BAILEY, A.R., CROW, T.J., JOHNSTONE, E.C. & OWEN, F. (1975). Platelet monoamine oxidase activity in patients with chronic schizophrenic illnesses untreated by neuroleptic drugs. *Br. J. clin. Pharmac.*, **2**, 380P.

GENTIL, V., GREENWOOD, M.H. & LADER, M.H. (1975). The effect of adrenaline on human platelet MAO activity. *Psychopharmacologia*, **44**, 187–190.

MELTZER, H.Y. & STAHL, S.M. (1974). Platelet monoamine oxdiase activity and substrate preferences in schizophrenic patients. *Res. Commun. Pathol. Pharm.*, **7**, 419–431.

MURPHY, D.L. & WYATT, R.J. (1972). Reduced monoamine oxidase activity in blood platelets from schizophrenic patients. *Nature (Lond.)*, **238**, 225–226.

ROBINSON, D.S., LOVENBERG, W., KEISER, H & SJOERDSMA, A. (1968). Effects of drugs on human blood platelet and plasma amine oxidase activity *in vitro* and *in vivo*. *Biochem. Pharmac.*, **17**, 109–119.

SHASKAN, E.G. & BECKER, R.E. (1975). Platelet monoamine oxidase in schizophrenics. *Nature (Lond.)*, **253**, 659.

---

## Role of lymphocytes in accumulation of fibrin in rabbit skin homografts

CATHERINE S. BACH &
M.K. JASANI

*Horsham Research Centre, CIBA Laboratories, Horsham, West Sussex, RH12 4AB.*

Indomethacin-like drugs relieve joint pain and stiffness in rheumatoid arthritis but do not reduce the swelling. Further insight into this problem has been obtained by investigating the mechanisms influencing the fibrin content of homografts, as suggested by Jasani, Lewis & Tweed (1974).

Fibrin accumulation was studied by administering iodinated $^{125}$I-labelled human fibrinogen (100 µCi in 1 ml water, i.v.) to New Zealand White rabbits bearing six homografts (from Norfolk donors) of the right hind limb and an equal number of autografts on the left leg. One homograft and its corresponding autograft were removed daily from day four. $^{125}$I-labelled fibrinogen was given on day five and a blood sample taken daily thereafter. Grafts were kept frozen (−20°C), homogenized (Jasani, 1973) and the water-insoluble radioactivity determined and expressed as a fraction of blood radioactivity according to Colvin & Dvorak (1975), thus minimizing inter-animal variations.

Figure 1a shows that the insoluble $^{125}$I-labelled fibrinogen content, although similar at first in both types of graft, began to increase significantly in the homografts following the appearance of cyanosis.

determined and expressed as a fraction of blood radioactivity according to Colvin & Dvorak (1975), thus minimizing inter-animal variations.

Figure 1a shows that the insoluble $^{125}$I-fibrinogen content, although similar at first in both types of graft,

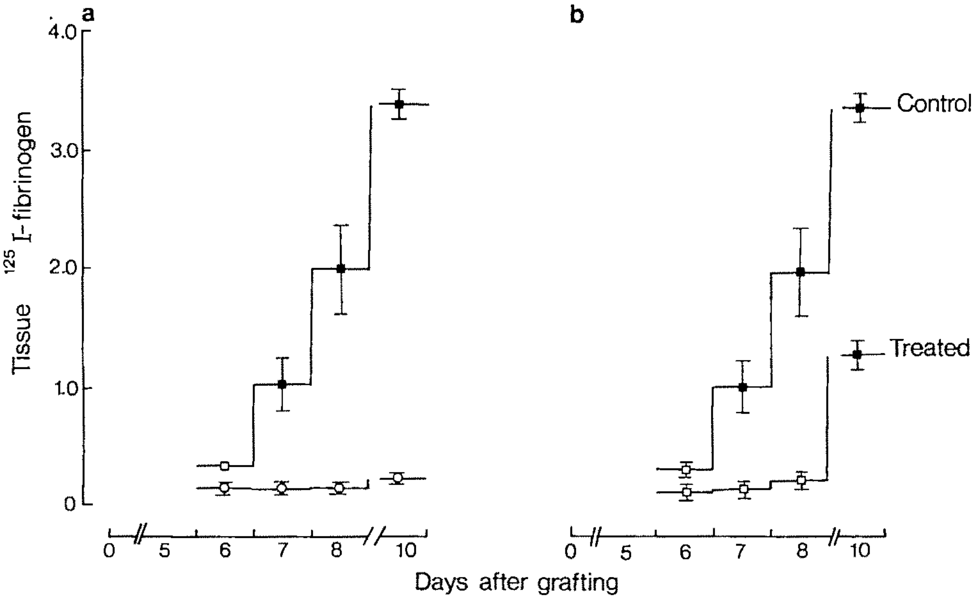


**Figure 1** Relationship between fibrin accumulation and the entry of circulating sensitized lymphocytes (SLs) as judged by the appearance of cyanosis in rabbit skin homografts.

Water insoluble $^{125}$I-labelled fibrinogen in (a) homografts (□) and autografts (O) and (b) control and cyclophosphamide (750 mg/animal) treated homografts. Full squares (■) denote that the homografts are cyanosed. Results are mean ± s.e. for three experiments.

began to increase significantly in the homografts following the appearance of cyanosis.

As cyanosis is initiated by the entry of circulating sensitized lymphocytes (Jasani, 1975), it is suggested that some factor released from these cells is responsible for the accumulation of fibrin in the homografts. This view is strengthened by the results of Figure 1b which show that the immunosuppressive agent cyclophosphamide, known to delay the development of circulating sensitized lymphocytes and swelling, prevented the insoluble fibrinogen content of homografts from increasing above the values found in the autografts, as long as cyanosis did not appear.

As a working hypothesis, failure of indomethacin-like drugs to arrest rheumatoid swelling may now be viewed as reflecting their inability to prevent the entry of circulating sensitized lymphocytes into the joint.

### References

COLVIN, R.B. & DVORAK, H.F. (1975). Role of the clotting system in cell-mediated hypersensitivity. *J. Immunol.*, **114**, 379–387.

JASANI, M.K. (1973). The usefulness of enzyme measurement for evaluation of pharmacological agents which modify the homograft reaction. *Biochem. Soc. Trans.*, **1**, 1043–1048.

JASANI, M.K. (1975). The importance of ACTH and glucocorticoids in rheumatoid arthritis. In *Clinics in Rheumatic Diseases*, ed., W.C. Dick & C.F. Pearson, Vol. 1 (2), p. 354. W.B. Saunders Ltd., London & Philadelphia.

JASANI, M.K., LEWIS, G.P. & TWEED, M.F. (1974). A new approach for studying the anti-rheumatic activity of indomethacin using the homograft reaction as a model for chronic immunologically-mediated inflammation. *Br. J. Pharmac.*, **50**, 475P.

## Accumulation of biogenic amines by rabbit erythrocytes

A.G.H. BLAKELEY & C.J.M. NICOL[1]

*Department of Pharmacology, The University, Glasgow, G12 8QQ.*

Adrenaline and noradrenaline (NA) enter cat and human erythrocytes during incubation at 37°C *in vitro* (Bain, Gaunt & Suffolk, 1937; Bain & Batty, 1956). Shanker, Nafpliotis & Johnson (1961) concluded that the entry of catecholamines and 5-hydroxytryptamine (5-HT) was by simple diffusion. Danon & Sapira (1972), disagreeing, proposed an active transport.

Blood was obtained from anaesthetized Dutch rabbits treated with heparin (1000 i.u./kg i.v.). An erythrocyte suspension in Krebs bicarbonate saline was prepared after removal of the white cells, platelets and plasma proteins by combined filtration through glass-wool and saline washes.

Samples of the erythrocyte suspension were incubated at 0°C or 37°C with $[^3H]$- or $[^{14}C]$-labelled NA, 5-HT or tyramine. The samples were then rapidly cooled in an ice-bath, spun (3100 *g* for 15 min) and the amount of radioactivity in the supernatant and packed erythrocyte layers determined by liquid scintillation counting. The activity in the red cells was calculated after correction for radioactivity trapped extracellularly.

Paper chromatography showed that only NA was broken down during incubation and that this was due to intracellular metabolism by catechol-O-methyltransferase. This breakdown was measured by column chromatography (Dubocovich & Langer, 1973).

At 37°C (−)-NA (1 µg/ml) entered the erythrocytes slowly and reached, after 4 h, a cell/medium concentration ratio of $2.13 \pm 0.05$ (s.e. of mean, $n = 12$). 5-HT entered faster, reaching a ratio of $2.75 \pm 0.05$ ($n = 19$) in 30 minutes. Tyramine achieved a ratio of $1.64 \pm 0.03$ ($n = 6$) within 5 minutes. Cooling to 0°C completely inhibited the entry of (−)-NA and 5-HT but had no effect on tyramine entry.

NA entry was stereoselective; the (−) isomer entering twice as fast as the (+) isomer.

(−)-NA entry obeyed Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ were respectively $6.6 \times 10^{-3}$M and $3.5 \times 10^{-7}$ mol min$^{-1}$ ml RBC$^{-1}$. (+)-NA and 5-HT also entered by saturable processes. The $K_m$ and $V_{max}$ for (+)-NA were $11.3 \times 10^{-3}$M and $3.6 \times 10^{-7}$ mol min$^{-1}$ ml RBC$^{-1}$, respectively and for 5-HT were $5.6 \times 10^{-3}$M and $2.1 \times 10^{-6}$ mol min$^{-1}$ ml RBC$^{-1}$, respectively. Tyramine entry did not saturate.

5-HT and (−)-NA, when presented simultaneously to the erythrocytes, competed for entry. In the presence of 5-HT the $K_m$ for (−)-NA entry was significantly ($P < 0.01$) increased to $10.5 \times 10^{-3}$M, while the $V_{max}$, $3.5 \times 10^{-7}$ mol min$^{-1}$ ml RBC$^{-1}$ was unaffected.

The amines were apparently concentrated in the cells. This was a result of binding to haemoglobin and a Donnan equilibrium across the membrane.

NA and 5-HT were therefore crossing the erythrocyte membrane by a process of carrier-mediated diffusion. Tyramine entered these cells apparently by simple diffusion.

This work was supported by the Rankin Medical Research Fund. CJMN was an MRC scholar.

### References

BAIN, W.A. & BATTY, J.E. (1956). Inactivation of adrenaline and noradrenaline by human and other mammalian liver *in vitro*. *Brit. J. Pharmacol.*, **11**, 52–57.

BAIN, W.A., GAUNT, W.E. & SUFFOLK, S.F. (1937). Observations on the inactivation of adrenaline by blood and tissues *in vitro*. *J. Physiol. (Lond.)*, **91**, 233–253.

DANON, A. & SAPIRA, J.D. (1972). Uptake and metabolism of catecholamines by the human red blood cell. *Clin. Pharmac. Ther.*, **13**, 916–922.

DUBOCOVICH, M. & LANGER, S.Z. (1973). Effects of flow-step on the metabolism of noradrenaline released by nerve stimulation in the perfused spleen. *Naunyn-Schmiedeberg's Archs. Pharmac.*, **278**, 179–194.

SCHANKER, L.S., NAFPLIOTIS, P.A. & JOHNSON, J.M. (1961). Passage of organic bases into human red cells. *J. Pharmac. exp. Ther.*, **133**, 325–331.

---

[1] Present address: Dept. of Physiology, University of St. Andrews.

# Effect of *p*-bromophenacyl bromide, an inhibitor of phospholipase $A_2$, on prostaglandin production by the guinea-pig uterus

SHEILA M. MITCHELL, N.L. POYSER & N.H. WILSON

*Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, Scotland.*

Membrane phospholipids are a probable source of arachidonic acid for prostaglandin synthesis, though this has still to be proven. Phospholipase $A_2$ ($PLA_2$) cleaves unsaturated fatty acids from phospholipids, so its inhibition should prevent prostaglandin production by tissues if phospholipids are involved. Para-bromophenacyl bromide (*p*-BPAB), an inhibitor of porcine pancreatic $PLA_2$ (Volwerk, Pieterson & de Haas, 1974) may be useful in studying arachidonic acid release from and prostaglandin synthesis by tissues. Its effect of these parameters has been examined *in vitro* on the guinea-pig uterus, a tissue which synthesizes predominantly prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from endogenous precursors when homogenized and incubated *in vitro* (Poyser, 1972).

Uteri from guinea-pigs in dioestrus were homogenized in 15 ml Krebs solution and incubated for 90 min in the absence or presence of a known concentration of *p*-BPAB. Each uterus was so divided so as to act as its own control. $PGF_{2\alpha}$ synthesis was inhibited, but not completely, by *p*-BPAB (Table 1). A re-direction of synthesis towards $PGE_2$ did not occur as its synthesis was also inhibited (63.0 ± 4.3%; $n = 5$) by *p*-BPAB (100 µg/ml). Metabolism of 9-[$^3$H]-$PGF_{2\alpha}$ (0.4 µCi) by the uterus was unaffected by *p*-BPAB (100 µg/ml) and remained undetectable ($n = 2$).

The release of free arachidonic acid during incubation of the guinea-pig uterus was also measured in the absence or presence of *p*-BPAB (100 µg/ml). Indomethacin (50 µg/ml) was present in the Krebs solution during this study. The post-homogenization, pre-incubation level (µg/100 mg tissue) of free arachidonic acid in the uterus was 1.6 ± 0.4 ($n = 5$), which rose to 4.5 ± 0.4 following incubation. This increase was inhibited by 75.9% with *p*-BPAB (the level of arachidonic acid being 2.3 ± 0.4) achieved possibly by inhibiting $PLA_2$. The amount of arachidonic acid available is, however, still far in excess of the amounts of prostaglandins normally synthesized by the uterus. It would appear that inhibition of arachidonic acid release does not account for the reduction in prostaglandin synthesis. Furthermore, the addition of exogenous arachidonic acid (10 µg/ml) to uterine incubates ($n = 5$) did not overcome the inhibition of prostaglandin synthesis produced by *p*-BPAB. Apparently this compound also prevents the conversion of arachidonic acid into prostaglandins.

This study was supported by the M.R.C.

**References**

POYSER, N.L. (1972). Production of prostaglandins by the guinea-pig uterus. *J. Endocr.*, **54**, 147–159.

VOLWERK, I.J., PIETERSON, W.A. & DE HAAS, G.H. (1974). Histidine at the active site of phospholipase $A_2$. *Biochemistry*, **13**, 1446–1454.

**Table 1** Effect of *p*-bromophenacyl bromide (*p*-BPAB) on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthesis by guinea-pig uterus homogenates *in vitro*.

| *Concentration of p-BPAB (µg/ml)* | *% Inhibition of $PGF_{2\alpha}$ synthesis (mean ± s.e.)* | *No. of repetitions (n)* |
|---|---|---|
| 1 | 6.6 ± 1.4 | 4 |
| 2 | 25.2 ± 7.3 | 4 |
| 5 | 38.4 ± 6.7 | 4 |
| 10 | 51.1 ± 7.0 | 3 |
| 25 | 53.0 ± 10.3 | 4 |
| 50 | 52.8 ± 5.2 | 4 |
| 100 | 68.0 ± 6.0 | 7 |
| 200 | 68.7 ± 6.0 | 5 |
| 400 | 77.0 ± 6.3 | 4 |

# Generation of thromboxane $A_2$-like activity from prostaglandin endoperoxides by polymorphonuclear leukocyte homogenates

S. BUNTING, G.A. HIGGS, S. MONCADA & J.R. VANE

*The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS.*

Rabbit aorta contracting substance (RCS) released from isolated perfused lungs of guinea pigs during anaphylaxis (Piper & Vane, 1969) is now thought to be a mixture of thromboxane $A_2$ ($TXA_2$) and prostaglandin endoperoxides ($PGG_2$, $PGH_2$; Hamberg, Svensson & Samuelsson, 1975; Bunting, Moncada & Vane, 1976). $TXA_2$ is generated enzymically from $PGG_2$ or $PGH_2$ by horse or human platelet microsomes (Bunting, Moncada, Needleman & Vane, 1975). $TXA_2$ is distinguished from endoperoxides by its shorter half life and its increased potency in contracting the rabbit aorta; moreover, while prostaglandin endoperoxides relax the mesenteric and coeliac arteries of the rabbit, $TXA_2$ is a powerful constrictor of both (Bunting, Moncada & Vane, 1976).

Rabbit peritoneal polymorphs (PMNs) release prostaglanin during phagocytosis (Higgs, McCall & Youlten, 1975). We have now shown that homogenates of these cells contain an enzyme which converts PG endoperoxides into a substance with increased RCS activity which contracts the rabbit coeliac artery and has a similar half life to $TXA_2$.

PMN leukocyte suspensions obtained by the method of Hirsch & Church (1960) were centrifuged (50 *g* for 10 min), washed once with 0.9% saline and resuspended in 5 ml Krebs bicarbonate solution at a concentration of $4–18 \times 10^7$ cells/ml. The cells were disrupted by freezing and thawing five times and homogenized in a glass homogenizer. The homogenates were then centrifuged at 100,000 *g* for 1 h and resuspended in 2 ml 100 mM Tris buffer (pH 7.5) prior to incubation with $PGG_2$ or $PGH_2$ (1 μg/ml). The biological activity of the incubation mixtures was assayed on spirally cut strips of rabbit aorta and rabbit coeliac artery, superfused at 10 ml/min with Krebs bicarbonate containing a mixture of antagonists (Gilmore, Vane & Wyllie, 1968) plus indomethacin (2 μg/ml). Changes in length of the assay tissues were detected by Harvard smooth muscle transducers and displayed on a Watanabe pen recorder.

Homogenates of PMNs were incubated with $PGG_2$ or $PGH_2$ for 2 min at 0°C to test for thromboxane synthetase activity. None was detected in resting PMNs but when they were incubated with killed bacteria (Pertussis vaccine, Burroughs Wellcome) for 1 h at 37°C prior to homogenization, increased RCS activity was generated and the coeliac artery contracted. This conversion was prevented by boiling the homogenate prior to incubation, or by addition of benzydamine (200 μg/ml) a drug which inhibits the formation of $TXA_2$ by horse platelet microsomes (Moncada, Needleman, Bunting & Vane, unpublished) but not indomethacin (10 μg/ml). Incubation mixtures at neutral pH and 0°C were rapidly extracted with dry ether; the ether was evaporated under nitrogen and the residue taken up in buffer. Extracts contained RCS activity with a half life of 10–11 min at 0°C, 1–2 min at room temperature and less than 1 min at 37°C; values similar to those described for $TXA_2$.

These results show that homogenates of phagocytosing PMNs generate thromboxane $A_2$-like activity. Thromboxane $B_2$, the metabolite of $TXA_2$ is chemotactic (Boot, Dawson & Kitchen, 1976); thus the production of thromboxanes by PMNs during phagocytosis could contribute to the inflammatory process.

## References

BOOT, J.R., DAWSON, W. & KITCHEN, E.A. (1976). The chemotactic activity of thromboxane $B_2$: a possible role in inflammation. *J. Physiol. (Lond.)*, in press

BUNTING, S., MONCADA, S., NEEDLEMAN, P. & VANE, J.R. (1975). Formation of prostaglandin endoperoxides and rabbit aorta contracting substance (RCS) by coupling two enzyme systems. *Br. J. Pharmac.*, **56**, 344P–345P.

BUNTING, S., MONCADA, S. & VANE, J.R. (1976). The effects of prostaglandin endoperoxides and thromboxane $A_2$ on strips of rabbit coeliac artery and certain other smooth muscle preparations. *Br. J. Pharmac.*, in press.

GILMORE, N., VANE, J.R. & WYLLIE, H.J. (1968). Prostaglandins released by the spleen. *Nature (Lond.)*, **218**, 1135–1140.

HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Nat. Acad. Sci., U.S.A.*, **72**, 2994–2998.

HIGGS, G.A., McCALL, E. & YOULTEN, L.J.F. (1975). A chemotactic role for prostaglandins released from polymorphonuclear leukocytes during phagocytosis. *Br. J. Pharmac.*, **53**, 539–546.

HIRSCH, J.G. & CHURCH, A.B. (1960). Studies of phagocytosis of group A streptococci by polymorphonuclear leukocytes *in vitro*. *J. exp. Med.*, **III**, 309–322.

PIPER, P.J. & VANE, J.R. (1969). The release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature (Lond.)*, **223**, 29–35.

# The effect of pulmonary metabolites of prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ on ADP-induced aggregation of human and rabbit platelets

J. WESTWICK (introduced by G.P. LEWIS)

*Department of Pharmacology, Royal College of Surgeons of England, London, WC2A 3PN.*

Prostaglandins (PGs) have been extensively examined as regulators of platelet activity since Kloeze (1967, 1969) demonstrated that $PGE_1$ was a potent inhibitor of platelet aggregation. Recently the pulmonary metabolites of various prostaglandins have been shown to have considerable biological activity, e.g. on the bronchioles (Dawson, Lewis, McMahon & Sweatman, 1974), uterus (Crutchley & Piper, 1976) and blood pressure (Änggard, 1966).

In the present study, a comparison has been made of nine pulmonary metabolites on ADP-induced aggregation of human and rabbit platelets. Human and rabbit platelet-rich plasma (PRP) were prepared by the method of Holmsen, Storm & Day (1972). The platelet counts were adjusted to 300,000/µl and 400,000/µl in human and rabbit PRP respectively. Platelet aggregation was measured as an increase in light transmission as described by Born & Cross (1963). In order to test the normal reactivity of the platelets, with every batch of PRP a dose-response curve to ADP was obtained. A concentration of $1.3 \times 10^{-6}$ ADP which produced about a 50% response, was selected for testing inhibition of aggregation. Following a 2 min pre-incubation period of the PRP to stabilize the temperature at 37°C, a constant volume (50 µl) of vehicle or prostaglandin (final concentrations $5 \times 10^{-9}$ M to $5 \times 10^{-5}$ M) was added, followed 3 min later by ADP. Each concentration of PG was tested 3–5 times and alternating control tests were made with vehicle alone.

These results were expressed as percentage of control response. Dose-response curves were plotted and the $IC_{50}$ values (µM) are shown in Table 1.

These findings confirm that in man and rabbit, $PGE_1$ is a potent inhibitor of aggregation. However, the 13,14-dihydro-$PGE_1$ is also active in both species and is more than twice as active as the parent $PGE_1$ in man. While in high concentrations $PGE_2$ inhibits aggregation, in lower concentrations it causes potentiation of ADP-induced aggregation in man and rabbit. On the other hand, 13,14-dihydro-$PGE_2$ potentiates aggregation only in man. The extent to which human platelets metabolize prostaglandins and the effect of the metabolites on other platelet function tests are to be investigated.

This work was generously supported by Ciba/Geigy. We wish to thank Dr J.E. Pike (Upjohn, Kalamazoo) for the supply of prostaglandins.

**References**

ÄNGGÅRD, E. (1966). The biological activities of three metabolites of prostaglandin $E_1$. *Acta physiol. scand.*, **66**, 509–510.

BORN, G.V.R. & CROSS, M.J. (1963). The aggregation of blood platelets. *J. Physiol. (Lond.)*, **168**, 178–185.

**Table 1** Comparison of prostaglandin metabolites on ADP-induced platelet aggregation.

| | *$IC_{50}$ values (µM)* | |
|---|---|---|
| *Prostaglandin* | *Rabbit* | *Man* |
| $PGE_1$ | 0.039 | 0.08 |
| 15-keto-$PGE_1$ | >50.0 | >50.0 |
| 13,14-dihydro-15-keto-$PGE_1$ | >50.0 | >50.0 |
| 13,14-dihydro-$PGE_1$ | 0.19 | 0.037 |
| $PGE_2$ | 28.0* | 13.0** |
| 15-keto-$PGE_2$ | >50.0 | >50.0 |
| 13,14-dihydro-15-keto-$PGE_2$ | >50.0 | >50.0 |
| 13,14-dihydro-$PGE_2$ | 50.0 | 20.0** |
| $PGF_{2\alpha}$ | N.T. | 10.0 |
| 15-keto-$PGF_{2\alpha}$ | | >50.0 |
| 13,14-dihydro-15-keto-$PGF_{2\alpha}$ | | >50.0 |
| 13,14-dihydro-$PGF_{2\alpha}$ | | >50.0 |

* 0.25–2.7 µM and ** 0.08–0.26 µM produce potentiation ($P < 0.05$) of platelet aggregation. N.T. = not tested.

CRUTCHLEY, D.J. & PIPER, P.J. (1976). Bioassay and thin layer chromatography of prostaglandins and their pulmonary metabolites. *Br. J. Pharmac.* (in press).

DAWSON, W., LEWIS, R.L., McMAHON, R.E. & SWEATMAN, W.J.F. (1974). Potent bronchoconstrictor activity of 15-keto-prostaglandin $F_{2\alpha}$. *Nature (Lond.)*, **250**, 331–332.

HOLMSEN, H., STORM, E. & DAY, H.I. (1972). Determination of ATP and ADP in blood platelets. *Analyt. Biochem.*, **46**, 489–501.

KLOEZE, J. (1967). Influence of prostaglandins on platelet adhesiveness and platelet aggregation. Nobel Symposium 2, Stockholm, June 1966. In *Prostaglandins*, ed. S. Bergstrom & B. Samuelsson, pp. 241–252. Stockholm: Almqvist & Wiksell.

KLOEZE, J. (1969). Relationship between chemical structures and platelet aggregation activity of prostaglandins. *Biochim. Biophys. Acta*, **187**, 285–292.

---

## Stimulation of platelets by bis-enoic prostaglandins

J.L. GORDON & D.E. MacINTYRE

*Department of Pathology, University of Cambridge.*

Prostaglandins (PGs) can stimulate or inhibit platelets: $PGE_1$ and $PGD_2$ are inhibitory (Kloeze, 1967; Smith, Silver, Ingerman & Kocsis, 1974), 11-deoxy-15-methyl-15RS-$PGE_2$ (Wy-17,186) stimulates aggregation directly (Fenichel, Stokes & Alburn, 1975), and $PGE_2$ stimulates or inhibits depending on the conditions (MacIntyre & Gordon, 1975).

We investigated the effects of $PGF_{2\alpha}$, $PGE_2$, Wy-17,186, 16,16-dimethyl-$PGE_2$, 15(R)-15-methyl-$PGE_2$ and 15(S)-15-methyl-$PGE_2$ methyl ester in human and pig platelet-rich plasma (PRP) as described previously (Gordon & Drummond, 1974; MacIntyre & Gordon, 1975). All these compounds except 15(R)-15-methyl $PGE_2$ induced platelet aggregation in pig heparinized PRP; minimum active concentrations (µg/ml) were respectively 2.0, 0.8, 0.6, 0.2, >100, 3.0.

In human citrated PRP, only 16,16-dimethyl-$PGE_2$ and Wy-17,186 induced substantial aggregation; 15(S)-15-methyl-$PGE_2$ methyl ester had a slight effect. At 3 µg/ml, 16,16-dimethyl-$PGE_2$ and Wy-17,186 released 40–70% of platelet granule constituents (measured by prelabelling with [$^{14}$C]-serotonin) and less than 10% of cytoplasmic constituents (measured by prelabelling with [$^3$H]-adenine). Aggregation and release induced by the methylated prostaglandins were inhibited by $PGD_2$, $PGE_1$, $PGE_2$, $PGF_{2\alpha}$ and 15(R)-15-methyl-$PGE_2$: against aggregation induced by ADP, $PGD_2$ and $PGE_1$ were similarly effective, but the other inhibitory PGs were much less active (Table 1).

These findings support the concept that blood platelets can provide a valuable model for studying the characteristics of cellular receptors for PGs. Methylation at positions 15 and 16, and the stereospecificity of this substitution, clearly affect the stimulatory or inhibitory potency of prostaglandins. Furthermore, the differential potency of inhibitory PGs against platelet aggregation induced by $PGE_2$ derivatives or ADP suggests that the site of action may be at the stimulatory receptor in the former case, but at a separate site (e.g. adenylate cyclase) in the latter.

This work was supported by grants from the M.R.C. and the Arthritis and Rheumatism Research Council. We thank Dr R.L. Fenichel (Wyeth) for the gift of Wy-17,186 and Dr J.E. Pike (Upjohn) for the gift of the other prostaglandins.

**Table 1** Inhibition of human platelet aggregation by prostaglandins

| | *$IC_{50}$ values (µg/ml)<br>Aggregating Agent* | | |
|---|---|---|---|
| *Prostaglandin* | *16,16-diMe-$PGE_2$ (3 µg/ml)* | *Wy-17,186 (3 µg/ml)* | *ADP (1 µM)* |
| $PGD_2$ | 0.06 | 0.07 | 0.01 |
| $PGE_1$ | 0.02 | 0.06 | 0.01 |
| $PGE_2$ | 0.20 | 0.80 | 10 |
| 15-Me-15(R)-$PGE_2$ | 50 | 45 | >100 |
| $PGF_{2\alpha}$ | 2.5 | 4.0 | >100 |

Results are mean values of triplicate determinations.

HT in PRP, but when 5-HT was incubated in human PRP for 1 min or longer, the inhibitory effect of ADP was increased. The mechanism by which ADP inhibits 5-HT uptake is not fully understood, but since it occurs at submicromolar concentrations of ADP, it may be of considerable physiological significance.

This study was supported by a research grant from the Arthritis & Rheumatism Council.

**References**

BORN, G.V.R. & GILLSON, R.E. (1959). Studies on the uptake of 5-hydroxytryptamine by blood platelets. *J. Physiol. (Lond.)*, **146**, 472–491.

BUCZKO, W., DE GAETANO, G. & GARATTINI, S. (1975). Effect of fenfluramine on 5-hydrotryptamine uptake and release by rat blood platelets. *Br. J. Pharmac.*, **53**, 563–568.

DA PRADA, M. & PLETSCHER, A. (1968). Isolated 5-hydroxytryptamine organelles of rabbit blood platelets: physiological properties and drug induced changes. *Br. J. Pharmac.*, **34**, 591–597.

DRUMMOND, A.H. & GORDON, J.L. (1976). Uptake of 5-hydroxytryptamine by rat blood platelets and its inhibition by adenosine-5-diphosphate. *Br. J. Pharmac.*, **56**, 417–421.

HORNG, J.S. & WONG, D.T. (1976). Effects of serotonin uptake inhibitor, Lilly 110140, on transport of serotonin in rat and human blood platelets. *Biochem. Pharmacol.*, **25**, 865–867.

SNEDDON, J.M. (1973). In *Progress in Neurobiology* ed. Kerkut, G.A. & Phillis, J.W. **Vol. 1**, pp. 151–198. Oxford: Pergamon Press.

---

## A further action of sodium cromoglycate

D.M. JACKSON & I.M. RICHARDS

*Fisons Ltd., Pharmaceutical Division, Research & Development Laboratories, Bakewell Rd., Loughborough, Leicestershire LE11 0QY.*

At the last meeting of the British Pharmacological Society at Chelsea College we described a model of reflex bronchoconstriction in the anaesthetized dog (Richards & Jackson, 1976). The activity of sodium cromoglycate (DSCG) has now been investigated using this model.

Beagle dogs of either sex and weighing 9–12 kg were used in this study. The dogs were initially sedated with thiopentone sodium (5–10 mg/kg i.v.) and then anaesthetized with chloralose (80 mg/kg i.v.). The dogs were respired at constant pressure with a Bird Mk. VII ventilator and airways resistance ($R$ ) and dynamic lung compliance (Cdyn) measured continuously. Bronchoconstriction was produced by allowing the dogs to inhale 4 breaths of a histamine aerosol of 10 µm mean particle size through a tracheal cannula. The concentration of histamine solution from which the aerosol was generated was selected so that a change in $R$ of 10–20 cm $H_2O$ $l^{-1}$ $s^{-1}$ was produced. The concentrations used were 0.0625%–0.25%. The reflex component of the induced bronchoconstriction was determined by bilateral vagal cooling.

Histamine challenges were given every 30 min, and when 4 consistent reflex bronchoconstrictions had been produced the effects of 4 breaths of an aerosol of DSCG of 10 µm mean particle size, generated from either 1% or 2% solutions, on this bronchoconstriction were investigated. (The DSCG was given 10 min prior to the next histamine challenge.) The results of this study are shown in Figure 1.

After 1% DSCG there was some reduction in the

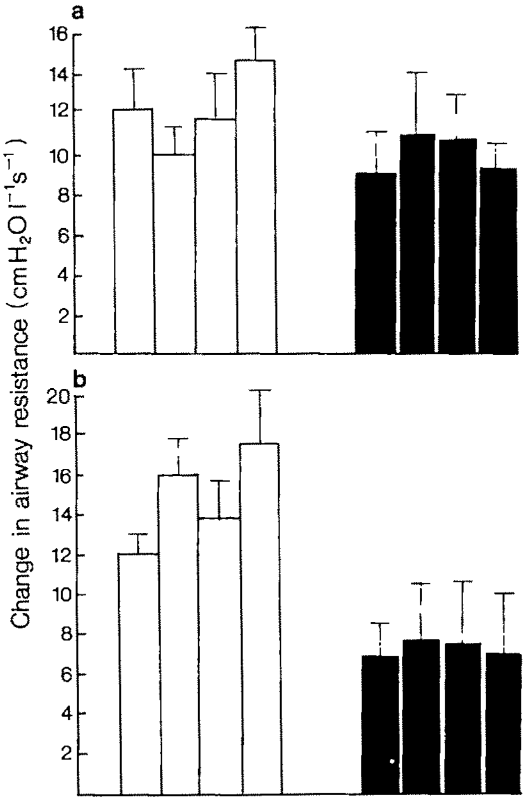


**Figure 1** Changes in airway resistance produced by inhalation of 4 breaths of histamine aerosol of 10 µm mean particle size. The open histograms are control values and the closed histograms are the responses after inhalation of 4 breaths of an aerosol of DSCG of 10 µm mean particle size generated from a 1% solution (a) and a 2% solution (b). (Results are from 6 and 4 dogs respectively. Bars are s.e. mean.)

response to histamine, although this was not statistically significant (Fig. 1A). Two per cent DSCG produced a significant reduction ($P<0.05$) in the induced bronchoconstriction which lasted for at least 2 h (Figure 1b). When DSCG (50 μg/kg) was given intravenously during a sustained reflex bronchoconstriction, complete reversal of the constriction was seen. The ability of the efferent autonomic nerves to produce a bronchoconstriction was checked throughout the experiment by direct electrical stimulation of the vagus nerves in the neck. DSCG, whether given by aerosol or intravenously did not inhibit the bronchoconstriction produced by direct electrical stimulation.

DSCG is not a bronchodilator or an antagonist of histamine or acetylcholine (Cox, Beach, Blair, Clarke, King, Lee, Loveday, Moss, Orr, Ritchie & Sheard, 1970) and it does not penetrate the central nervous system to any degree (personal communication from Dr B. Clarke, Dept. Metabolic Studies, Fisons Ltd.).

We concluded, therefore, that DSCG reduced the bronchoconstriction to histamine aerosol, in our experiments, probably by desensitizing the lung irritant receptors which are thought to be responsible for the initiation of the reflex bronchoconstriction to histamine aerosol (Sellick & Widdicombe, 1971).

**References**

COX, J.S.G., BEACH, J.E., BLAIR, A.M.J.N., CLARKE, A.J., KING, J., LEE, T.B., LOVEDAY, D.E.E., MOSS, G.F., ORR, T.S.C., RITCHIE, J.T. & SHEARD, P. (1970). Disodium Cromoglycate (Intal). *Adv. Drug Res.*, **5**, 115–193.

RICHARDS, I.M. & JACKSON, D.M. (1976). An investigation of histamine aerosol induced reflex bronchoconstriction in the anaesthetized dog. *Br. J. Pharmac.*, **57**, 438–439P.

SELLICK, H. & WIDDICOMBE, J.G. (1971). Stimulation of lung irritant receptors by cigarette smoke, carbon dust and histamine aerosol. *J. appl. Physiol.*, **31**, 15–19.

---

## The effects of corticosteroids on the responses of the anococcygeus and gastrocnemius muscles to nerve stimulation in the pithed rat

A. GIBSON[1], D. POLLOCK & J.C. SPENCE

*Department of Pharmacology, University of Glasgow*

Corticosteroids have been used to treat myasthenia gravis (Seybold & Drachman, 1974). The mechanism by which corticosteroids improve neuromuscular functioning in myasthenia is unknown but could be an immunosuppressive action on an auto-immune mechanism, which may cause this disease (Simpson, 1960). Since corticosteroids also have diverse effects on cardiac and smooth muscle (Thorp & Cobbin, 1967; Gibson & Pollock, 1975), this study considered the possibility that corticosteroids might have actions in striated muscle unconnected with an immune defect.

This study sought to determine whether corticosteroids could modify the responses of the rat gastrocnemius muscle to nerve stimulation. For comparison, the responses of a smooth muscle were also investigated. The smooth muscle chosen was the anococcygeus because its responses to agonists *in vitro* were enhanced by treatment of rats with corticosteroids (Gibson & Pollock, 1975). The responsiveness of these two muscles was investigated using the pithed rat preparation, in which a movable electrode was introduced into the spinal canal (Gillespie, MacLaren & Pollock, 1970). This technique permitted electrical stimulation of the spinal motor outflows to the gastrocnemius and anococcygeus in the same rat.

Male Wistar rats were treated with corticosterone (20 mg kg$^{-1}$ day$^{-1}$, i.p. for 5 days) or prednisolone (20 mg kg$^{-1}$ day$^{-1}$, i.p. for 4 days) or either saline or ethyl oleate as a control. The effects of reserpine (1 mg/kg, i.p. 20 h before pithing), which releases ACTH and therefore raises blood corticosteroid levels, were also investigated both in intact and in adrenalectomized rats. The rats were anaesthetized with trichloroethylene, respired artificially and pithed by the method of Gillespie, MacLaren & Pollock (1970). The spinal motor outflows were stimulated with supramaximal voltage using a Palmer stimulator. Responses of the muscles were recorded isometrically with Grass strain gauges and a Devices pen recorder. When the responses of the anococcygeus muscle were recorded, the rats received pancuronium bromide (2 mg/kg i.v.) to prevent interference by the contractions of the voluntary muscles.

Corticosterone and prednisolone increased the force of contraction of the single twitch and tetanic responses of the gastrocnemius muscle but the ratio of the tensions developed during these two responses was unchanged from control values. The corticosteroids impaired the ability of the gastrocnemius muscle to maintain a tetanus. Reserpine also increased the tension developed by the gastrocnemius muscle. This effect was absent in adrenalectomised rats. Corticosterone also increased the responses of the anococcygeus muscle to electrical stimulation. This occurred especially at low frequencies of stimulation. These results indicate that corticosteroids can enhance the responsiveness of normal striated muscle to electrical stimulation of the motor nerves. The mechanisms underlying this effect are unknown but may involve either presynaptic changes or perhaps, more likely, postsynaptic changes.

---

[1] Present Address: Chelsea College, London.

### References

GIBSON, A. & POLLOCK, D. (1975). The involvement of corticosteroids in the supersensitivity produced in the rat by morphine withdrawal, thyroidectomy or a single dose of reserpine. *J. Pharmac. exp. Ther.*, **192**, 390–398.

GILLESPIE, J.S., MacLAREN, A. & POLLOCK, D. (1970). A method of stimulating different segments of the autonomic outflow from the spinal column to various organs in the pithed cat and rat. *Br. J. Pharmac.*, **40**, 257–267.

SEYBOLD, M.E. & DRACHMAN, D.B. (1974). Gradually increasing doses of prednisolone in myasthenia gravis. *New Eng. J. Med.*, **290**, 81–84.

SIMPSON, J.A. (1960). Myasthenia gravis: a new hypothesis. *Scot. Med. J.*, **5**, 419–436.

THORP, R.H. & COBBIN, L.B. (1967). In *Cardiac Stimulant Substances*, pp. 234–236. New York: Academic Press.

---

# The action of metiamide in anaphylaxis *in vivo* in the guinea-pig

P. GOADBY & M. ANN LITTLE

*Department of Pharmacology, School of Pharmacy, Sunderland Polytechnic, Sunderland, SR1 3SD.*

Bartosch, Feldberg & Nagel (1932) first produced evidence that histamine was released from guinea-pig lungs after anaphylactic shock. Although histamine has been shown to interact with at least two types of receptor (Ash & Schild, 1966), most investigations of the role of histamine in anaphylaxis have studied only $H_1$-receptors. However, recent work has shown that $H_2$-receptors may be involved in immune reactions in basophil leucocytes and in the heart (Capurro & Levi, 1973; Chand & Eyre, 1975). In this study the effect of the $H_2$-receptor antagonist, metiamide (Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973) in anaphylaxis in the guinea-pig was investigated.

Guinea-pigs were sensitized to egg albumen by its injection (100 mg i.p. and 100 mg s.c.) 3 weeks before challenge. Exposure to an aerosol of 1% egg albumen produced an anaphylactic reaction characterized by dyspnoea and cough. The onset times of anaphylactic symptoms in animals pretreated with metiamide (10 mg/kg and 100 mg/kg s.c.) were 144.3 ± 51.0 s and 78.8 ± 13.3 s respectively and were not significantly different from those of control animals ($P<0.05$). Mepyramine (1 mg/kg i.p.) extended the time of onset of anaphylactic symptoms (551.9 ± 88.7 s) and the protection was not significantly altered by addition of 10 mg/kg metiamide (541.3 ± 94.0 s, $P<0.05$).

The aerosol method of studying anaphylaxis is very subjective and only gross effects are seen. Therefore, guinea-pigs anaesthetized with 60 mg/kg pentobarbitone sodium were prepared for the recording of respiratory overflow volume by the method of Konzett & Rössler (1940) as modified by Lessin & Kramer (1969). Heart rate and blood pressure were also monitored by means of a rate meter and pressure transducer. Histamine (0.25 μg–8.0 μg) caused increases in air overflow volume of 8.4–108.4%. The increases were blocked by mepyramine (0.05–5 mg/kg) but unaffected by metiamide (10 μg kg$^{-1}$ min$^{-1}$ i.v.). An increase in heart rate of 8.0 ± 3.1 to 51.1 ± 7.2 bts/min was produced by histamine between doses 2 μg and 16 μg. Mepyramine (5.0 mg/kg) produced a shift to the right of the dose response curve (dose ratio = 2.72) and mepyramine (5 mg/kg) plus metiamide (10 μg kg$^{-1}$ min$^{-1}$) caused a further shift to the right of the curve. (Dose ratio = 13.34.)

Injection of egg albumen (1 mg i.v.) into sensitized anaesthetized guinea-pig gave maximal increases in air overflow volume accompanied by an increase in heart rate and arterial blood pressure and death followed in 6 to 7 animals within 6 ± 0.83 minutes. Pretreatment with mepyramine (2.5 mg/kg), metiamide (50 μg kg$^{-1}$ min$^{-1}$) or mepyramine and metiamide in combination increased the number of survivors ($^{5}/_{5}$, $^{4}/_{5}$, $^{5}/_{5}$, respectively). Close inspection of the results suggested that metiamide may have been protecting the guinea-pigs by an action upon the heart. This is consistent with the theory that stimulation of $H_2$-receptors in the heart plays an important role in anaphylactic death.

### References

ASH, A.S.F. & SCHILD, H.O. (1966). Receptors mediating some actions of histamine. *Br. J. Pharmac. Chemother.*, **27**, 427–439.

BARTOSCH, R., FELDBERG, W. & NAGEL, E. (1932). Die Friewerden eines histaminähnlichen Stoffes bei der Anaphylaxie des Meerschweinchens. *Pflugers. Arch. ges. Physiol.*, **230**, 129–153.

BLACK, J.W., DUNCAN, W.A.M., EMMETT, J.C., distribution of histamine receptor sub-types. *Agents and Actions*, **5/4**, 277–295.

KONZETT, H. & RÖSSLER, R. (1940). Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur.

*Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*, **195**, 71–74.

LESSIN, A.W. & KRAMER, R.L. (1969). The bronchodilator action of analeptics in the guinea-pig. *J. Pharm. Pharmac.*, **21**, 309–313.

GANELLIN, C.R., HESSELBO, T., PARSONS, M.E. & WYLLIE, J.H. (1973). Metiamide—an orally active histamine $H_2$-receptor antagonist. *Agents and Actions*, **3/3**, 133–137.

CAPURRO, N. & LEVI, R. (1973). Anaphylaxis in the guinea-pig isolated heart: selective inhibition by burimamide of the positive inotropic and chronotropic effects of released histamine. *Br. J. Pharmac.*, **48**, 620–628.

CHAND, N. & EYRE, P. (1975). Classification and biological

---

# Factors regulating the time-course of the relaxation of rabbit aorta strips after contraction by angiotensin II

D. REGOLI, J. ST-LOUIS[1] & the late W.K. PARK

*Centre Hospitalier de l'Universite de Sherbrooke, Quebec, Canada*

It has been shown (Regoli & St-Louis, 1975) that the myotropic response of rabbit aorta strips (RAS) to angiotensin II is diminished in the absence of $Ca^{2+}$ and restored when $Ca^{2+}$ (1.5 mM) is readmitted to the physiological salt solution (PSS). The effect of $Ca^{2+}$ upon the angiotensin II response is still observed for several minutes after the angiotensin II infusion has been stopped, showing that angiotensin II continues to stimulate the receptors for several minutes after washing. Some factors regulating this continued myotropic response have now been investigated.

Helical RAS 2 cm long were equilibrated for 3 h under 3 g tension in a cascade system and superfused with a tris buffered PSS (van Breeman, Farinas, Gerba & McNaughton, 1972) maintained at 37°C. The drugs were applied by infusion in the PSS and isometric contractions were recorded by a Grass FTO3C force transducer.

A steady submaximal contraction was elicited with angiotensin II ($4.5 \times 10^{-9}$ M) and the rate of relaxation of the subsequent tension decrease was observed when angiotensin II or $Ca^{2+}$ or both were removed from the PSS. $RT_{50}$ values (time for 50% relaxation, Kalsner, 1975) were $7.1 \pm 0.3$ min following angiotensin II removal; $4.5 \pm 0.2$ min following $Ca^{2+}$ removal; and $3.8 \pm 0.2$ min following simultaneous removal of both ($n = 8$). Regoli & St-Louis (1975) have shown that changes in calcium concentration do not alter the receptor binding of angiotensin II but interfere with the magnitude of the contraction. The present results therefore show that interference with the contractile mechanism can increase the rate of relaxation.

When a 100-fold excess ($5 \times 10^{-7}$ M) of a potent competitive angiotensin II antagonist (8-Gly-angiotensin II; Regoli, Park & Rioux, 1974) was added during the angiotensin II infusion, there is again a decrease in $RT_{50}$ to $3.8 \pm 0.1$ min ($n = 12$). Hence the $RT_{50}$ value can also be decreased by promoting the dissociation of angiotensin II from its receptor (Rioux, Park & Regoli, 1975).

When the steady contraction was caused by analogues of angiotensin II (Regoli *et al.*, 1974) the $RT_{50}$ value for the relaxation after removing the compound from the PSS was correlated with the $pD_2$ value of each compound. When the antagonist 8-Gly-angiotensin II was added, the $RT_{50}$ values for these analogues were markedly reduced and were well correlated with the $pD_2$ value of each compound, $r = 0.928$ ($n = 6$ to 8 for each compound). The response to $Ca^{2+}$ readmission 30 s and 4 min after interrupting the infusion of these same analogues of angiotensin II in $Ca^{2+}$ free PSS, decreased with decreasing $pD_2$ value of the compounds. These last results suggest that the relaxation, and the residual contraction observed after adding $Ca^{2+}$ to the PSS, are highly dependent on the rate of dissociation of the agonist compound from the angiotensin II receptor.

The results presented here support the conclusion of Regoli & St-Louis (1975) that angiotensin II continues to stimulate the receptors several minutes after washing, and suggest that the dissociation of angiotensin II from its receptors is a rate-limiting step for the decrease in tension when angiotensin II is removed from the superfusing PSS.

DR is an associate of MRC Canada and JStL has a fellowship of CRS Quebec.

### References

KALSNER, S. (1975). Role of extraneuronal mechanisms in the termination of contractile responses to amines in vascular tissue. *Br. J. Pharmac.*, **53**, 267–277.

REGOLI, D., PARK, W.K. & RIOUX, F. (1974). Pharmacology of Angiotensin. *Pharmac. Rev.*, **26**, 69–123.

REGOLI, D. & ST-LOUIS, J. (1975). Influence of $Ca^{2+}$ and $Na^+$ on the affinities and intrinsic activities of Angiotensin and noradrenaline in rabbit aorta strips. *Fed. Proc.*, **39**, 770.

RIOUX, F., PARK, W.K. & REGOLI, D. (1975). Role of C-terminal group for the biological activities of angiotensin. *Can. J. Physiol. Pharmacol.*, **53**, 383–391.

VAN BREEMEN, C., FARINAS, B.R., GERBA, P. & McNAUGHTON, E.D. (1972). Excitation contraction coupling in rabbit aorta. Studied by the lanthonium method for measuring cellular calcium influx. *Circ. Res.*, **30**, 44–57.

---

[1] Present address: Wellcome Research Laboratories, Beckenham, Kent, U.K.

## $K^+$-induced relaxations of the rat anococcygeus muscle

A. GIBSON & T.A. JAMES

*Department of Pharmacology, Chelsea College, University of London, London, SW3 6LX.*

The actions of potassium chloride (KCl) on the rat anococcygeus muscle are complex (Gibson & Pollock, 1973). On the resting muscle, KCl produces dose-dependent contractions, which are partly due to release of endogenous noradrenaline (NA) and partly to a direct depolarisation of the muscle membrane. However, when the tone of the muscle is raised, by acetylcholine (ACh) or guanethidine, KCl now produces dose-dependent relaxations.

Two possibilities might explain these relaxations produced by KCl. Firstly, since KCl caused the release of motor transmitter (NA) it was possible that the relaxations were due to release of the as yet unknown inhibitory transmitter thought to exist in this tissue (Gillespie, 1972). Alternatively, it has been suggested that KCl might inhibit muscular activity by stimulating Na/K ATPase activity on the muscle membrane (Johns & Paton, 1974; Shibata, Fukada & Kurahashi, 1973) thus producing hyperpolarization and consequently relaxation. The purpose of the present study, therefore, was to determine which, if either, of the above mechanisms might explain KCl-induced relaxation of the rat anococcygeus muscle.

Adult male Wistar rats were killed by stunning and exsanguination. The two anococcygeus muscles were dissected (Gillespie, 1972) and suspended in oxygenated Krebs bicarbonate solution (36°C). To observe muscle relaxations to KCl the tone was first raised by addition of ACh (40 µM), normally in the presence of phentolamine (1 µM).

As found previously, KCl produced dose-related relaxations of the contracted anococcygeus muscle. Other $K^+$ salts (bicarbonate and tartrate) also relaxed the muscle, while NaCl was ineffective suggesting that the active ion was $K^+$. Dose-response curves for the contractile and relaxant actions of KCl were obtained, and these suggested that the muscle was more sensitive to the relaxant effects of KCl.

The $K^+$-induced relaxations were unaffected by addition of ouabain to the bathing medium (100 µM for 30 min). However, the relaxations were completely, but reversibly, abolished by tetrodotoxin (5 µg/ml). The local anaesthetics procaine or cocaine (both 500 µg/ml) also abolished $K^+$-induced relaxations, although they did not abolish the response to ACh, nor the relaxation following washout of agonist. Cooling the muscle to 10°C had a similar effect to that of the local anaesthetics.

In conclusion these experiments suggest that $K^+$-induced relaxations of the rat anococcygeus muscle are not due to stimulation of muscle Na/K ATPase, but that they are neurally-mediated and are probably due to release of the unknown inhibitory transmitter, the inhibitory nerves being more sensitive to the actions of $K^+$ than the motor nerves.

### References

GIBSON, A. & POLLOCK, D. (1973). The effects of drugs on the sensitivity of the rat anococcygeus muscle to agonists. *Br. J. Pharmac.*, **49**, 506–513.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmac.*, **45**, 404–416.

JOHNS, A. & PATON, D.M. (1974). Inhibitory effect of sodium pumping on spontaneous contractility of rabbit myometrium. *Can. J. Physiol. Pharmac.*, **52**, 786–790.

SHIBATA, S., FUKADA, H. & KURAHASHI, K. (1973). The inhibitory action of potassium on the mechanical and membranal activities of taenia from guinea-pig caecium. *Life Sci.*, **12**, 425–431.

---

## How can one set multiple choice examinations of equal difficulty from year to year?

R.W. FOSTER

*Pharmacology Department, Manchester University.*

The method which I am exploring attempts to predict the outcome of an examination as a normally distributed relationship between proportion of the candidates and score in the examination. The scoring system used is: correct +1, incorrect −1/(A-1), no answer 0. The mean score and variance of scores are obtained as in Figure 1.

The method used predictively makes three assumptions

(1) that $P_i$ can be estimated from the scores on question i attained by previous classes of candidates (and similarly $Q_i$ and $1-P_i-Q_i$).

$$\text{MEAN SCORE} = \sum_{i=1}^{N} P_i - \frac{Q_i}{A_i - 1}$$

$$\text{VARIANCE} = \text{VAR}_I\text{Y} + 2 \sum_{i=1}^{N} \sum_{\substack{j=1 \\ i \neq j}}^{N} R_{ij}\sqrt{\text{VARX}_i \times \text{VARX}_j}$$

$$\text{where VAR}_I\text{Y} = \sum_{i=1}^{N} \text{VARX}_i = \sum_{i=1}^{N} P_i\,(1-P_i) + \frac{2P_iQ_i}{A_i-1} + \frac{Q_i(1-Q_i)}{(A_i-1)^2}$$

where i or j = question number
N = total number of questions
P = probability of occurrence of correct answer
Q = probability of occurrence of incorrect answer
A = number of answer options
R = correlation coefficient

**Figure 1**

(2) that $R_{ij}$ can be estimated from an average of the correlation coefficients between the scores on every question and those on every other question obtained in previous examinations.

(3) that the distribution of candidates' scores on the whole examination can be estimated by a normal distribution. The current tests of the validity of these assumptions will be presented together with examples of comparisons between predicted and attained distributions.

Standardization from year to year would involve substitution of certain questions in the proposed examination until a prediction indicating the required difficulty emerged.

I am grateful to A.C.C. Gibbs for help in deriving the equations of Figure 1.

---

## Comparisons of performance in pharmacology examinations using multiple choice and essay questions

J.R.S. HOULT

*Department of Pharmacology, King's College, London WC2R 2LS*

Multiple choice questions (MCQ) have established a place alongside traditional essay questions in examinations in preclinical and clinical medicine. Ideally, the MCQ examination should be used to complement rather than to replace essay question papers, since the use of both types of examination explores a wider range of qualities of the candidates than if a single examining technique is used. Thus, a fairer and more comprehensive evaluation of the candidates may be made.

The automated marking and assessment methods used in our MCQ examinations have been demonstrated previously to the Society (Hoult, 1974) and the data presented here concern the results of six pharmacology examinations taken by preclinical medical students. Each examination comprises essay and MCQ sections. The essays are marked on a 'close-marking' scale (pass mark = 50) and the MCQ scores are adjusted to give a pass mark of 50. Individual candidate scores and the results of the whole group for the MCQ and essay sections have been compared to see if the two types of examination provide similar information about the abilities of the candidate.

The twelve frequency histograms of the scores of

**Table 1** Scores in multiple choice questions (MCQ) and essay sections of six preclinical pharmacology examinations

| *Examination* | *Number of Candidates* | *Essay scores** *%* | *MCQ scores** *%* | *% candidates with similar scores in each section†* |
|---|---|---|---|---|
| A | 119 | 57.9 ± 0.6 | 59.5 ± 1.0 | 71.4 |
| B | 103 | 52.0 ± 1.0 | 59.3 ± 0.9 | 71.8 |
| C | 109 | 52.4 ± 1.1 | 61.7 ± 0.9 | 57.8 |
| D | 114 | 55.0 ± 0.7 | 56.0 ± 0.8 | 83.3 |
| E | 122 | 55.0 ± 0.6 | 59.2 ± 0.8 | 74.6 |
| F | 115 | 56.9 ± 0.8 | 58.9 ± 0.9 | 56.5 |

* Results expressed as mean ± s.e.
† Scores on MCQ and essay sections differing by less than 10%.

the whole group for both sections of the six examinations all show scores which are normally distributed about a mean approximately one standard deviation above the pass mark. In each examination, the mean MCQ score of the whole group is slightly higher than the mean essay score (Table 1). Inspection of the histograms shows that the distribution of essay scores is slightly skewed, covering a wider range of scores at the lower end and a smaller range at the top end of the scale. Table 1 shows that, although most candidates obtained scores for the two sections of the examination which differed by less than 10%, there is a large goup of candidates with poor score correlations in the two tests.

These data show that the MCQ and essay sections of the examinations yield essentially similar information about the overall range of abilities of the candidates, and that many candidates achieve similar scores in both sections. However, there is an important group of candidates who score very differently in the two types of examinations. The existence of this group justifies the use of both types of assessment.

### Reference

HOULT, J.R.S. (1974). Fully automated multiple choice question marking. *Br. J. Pharmac.*, **52**, 154P.

---

## Learning from and attitudes to routine tape-slide teaching

J.M. FOY

*School of Pharmacology, University of Bradford, Bradford BD7 1DP*

The use of slides in the teaching of medical and allied subjects is of very long standing. Linking audio-tape with slides to provide a single educational unit is of more recent origin although in some institutions the method has been in use for about a decade (Amos, Duncan, Gilder, Hall & Smart, 1969; Holloway, 1964). Furthermore, the Medical Recording Service Foundation of the Royal College of General Practitioners has developed a large catalogue of recordings illustrated by slides which is extensively used and covers a wide range of subjects (Graves, 1971). This illustrates that even at the postgraduate level, where a helpful source of information exists, demand for it will be forthcoming, although research information on the efficacy of the service is lacking. Nevertheless, the use of tape-slide presentations in pharmacology teaching programmes, although accelerating, has not yet become routine in most courses perhaps because of the effort involved in preparation but also because of doubts regarding effectiveness and student acceptability once the novelty of the method has diminished. Indeed, most of the investigation into the efficacy of information retention have taken place in circumstances where the tape-slide method has been singled out for special attention either because the class was divided into special groups (Harden, Dunn, Holroyd, Lever, Lindsay & Wilson, 1969), or because special tests were administered (Birn & Christophersen, 1973).

Although not used very extensively tape-slide instruction has been a routine part of pharmacology teaching at Bradford for a number of years. The method of preparation and presentation of the tape-slides will be outlined. The results from computer marked objective tests given during the course reveal that compared with lecture based topics, answers arising from tape-slide generated material are between 3% and 12% better depending on the point of comparison. The majority of students expressed a desire for more such programmes. The main reported disadvantages of the method are connected with speed of presentation or technical difficulties (although given a choice students at another centre preferred automatic equipment in study booths; Harden, Stevenson, Lever, Holroyd & Wilson, 1975). But they readily appreciated the convenience of the method including the facility to repeat and ease of access and availability. A sample of the type of tape-slide in use will be available as a demonstration.

**References**

AMOS, S., DUNCAN, C.J., GLIDER, R.S., HALL, R. & SMART, G.A. (1969). Tape-slide programmes in medical education at the University of Newcastle-upon-Tyne. *Brit. J. med. Educ.*, **3**, 362–368.

BIRN, H. & CHRISTOPHERSEN, E. (1973). Evaluation of a course in surgical anatomy with special reference to the value of self-instruction. *Brit. J. med. Educ.*, **7**, 34–39.

GRAVES, J. (1971). Experience with a tape-slide service. *Scot. Medical J.*, **16**, 39–42.

HARDEN, R.McG., DUNN, W.R., HOLROYD, C., LEVER, R., LINDSAY, A. & WILSON, G.M. (1969). An experiment involving the substitutions of tape-slide programmes for lectures. *Lancet*, **1**, 933–935.

HARDEN, R.McG., STEVENSON, M., LEVER, R., DUNN, W., HOLROYD, C. & WILSON, G.M. (1975). Tape/slide programmes for independent study. *Br. J. med. Educ.*, **9**, 170–175.

HOLLOWAY, P.J. (1964). A test of the use of a teaching aid for the instruction of undergraduate dental students in operative techniques. *The Dental Practitioner*, **14**, 375–377.

---

## The teaching of pharmacology to undergraduate pharmacy students

J.M. FOY & JUDITH SENIOR

*School of Studies in Pharmacology, University of Bradford, Bradford, BD7 1DP*

It has been stated that learning is enhanced by feedback (Miller, 1967); this is true not only for the students studying in academic institutions but also for staff involved in teaching them. Students attending the School of Pharmacy, University of Bradford are frequently asked to complete questionnaires concerning the teaching methods and content of pharmacology courses (Cooper & Foy, 1967) but this is the first attempt we have made to evaluate the course by means of a questionnaire sent out to pharmacy graduates. The analysis was on a much smaller scale than the one on medical pharmacology recently reported from an American centre (Burford & Stritter, 1974).

The questionnaire was simple and divided into two main parts, the first part being involved with the occupation of the ex-student since graduation and the second part devoted to an evaluation of the pharmacology section of the pharmacy course. The questionnaire was sent to all persons (212) who graduated during the years 1972–74 from this School of Pharmacy, 51% of the forms were completed and returned for analysis.

The undergraduate course in pharmacology for pharmacy students in this University can be divided into two main sections, namely, experimental pharmacology in the second year and applied pharmacology in the third year of studies. The experimental pharmacology course introduces the student to the fundamental principles of the science of pharmacology and is supported by a practical programme which demonstrates the mechanism of drug action. The course in applied pharmacology has been described fully eslewhere (Foy, Hicks, Leach & Senior, 1972) and is designed to prepare the student for a role in a clinical multi-disciplinary team. Approximately 50% of the applied pharmacology section is composed of the study of clinical pharmacology. Results from the completed questionnaires show that the pharmacology component of the pharmacy course was rated at 3.5 on a 'completely satisfactory' 5 — > 1 'inadequate' scale. The relevance of the pharmacology course to the work currently being undertaken by the graduate varies from 3.6 on a 'relevant' 5 — >1 'irrelevant' scale for 1974 and 1973 graduates to 2.9 for 1972 graduates. The survey calls for more emphasis in the course to be placed on drug interactions, applied pharmacology and therapeutics, toxicology and pathology. Topics which are seen as expendable to allow expansion in the above areas are pharmacodynamics and pharmacokinetics. In a previous survey sent to all types of recent graduates in pharmacology the most frequent criticism of the components of the pharmacology course was also lack of clinical teaching on the use of drugs (Bakhle, Straughan & Webster, 1974).

The results from this latest 1976 survey to be extended in the communication show that a further emphasis towards clinical pharmacology may be desirable if the undergraduate course for pharmacists is to result in a more adequate pharmacological training.

**References**

BAHLE, Y.S., STRAUGHAN, D.W. & WEBSTER, R.A. (1974). Second report on the organization of pharmacology in Great Briatin. *Br. J. Pharmac.*, **50**, 163–175.

BURFORD, H.J. & STRITTER, F.T. (1974). Evaluation of a teaching program in medical pharmacology, *J. Med. Educ.*, **49**, 236–244.

COOPER, B. & FOY, J.M. (1967). Students' attitudes to learning from programs, lectures and textbooks. *Amer. J. Pharm. Educ.*, **31**, 461–466.

FOY, J.M., HICKS, R., LEACH, G.D.H. & SENIOR, J. (1972). The teaching of applied pharmacology. *Pharm. J.*, **209**, 16–18.

FOY, J.M. & SENIOR, J. (1974). Do 'A' levels really matter? *Pharm. J.*, **213**, 379–381.

MILLER, G.E. (1967). Educational science and education for medicine. *Brit. J. Med. Educ.*, **1**, 156–159.

---

## Career patterns in science graduate pharmacologists

P.B. MARSHALL & I.H. STEVENSON

*Department of Pharmacology and Therapeutics, University of Dundee*

To meet the increasing demand for pharmacologists in both universities and the pharmaceutical industry, several universities have over the past 25 years established science-based degrees in pharmacology.

**Table 1** Career patterns in science graduate pharmacologists

| | *Ordinary B.Sc.* | *Honours B.Sc.* | *M.Sc./Ph.d.* |
|---|---|---|---|
| No. of students | 181 | 53 | 25 |
| *After graduation* | | | |
| Honours B.Sc. (other subjects) | 17 | — | — |
| M.Sc./Ph.D. | 2 | 26 | — |
| Medicine/Dentistry | 3 | 5 | 1 |
| University teaching/research | 0 | 2 | 15 |
| School teaching | 8 | 1 | — |
| Pharmaceutical/chemical industry | 13 | 8 | 8 |
| Laboratory-based occupations | 8 | 4 | — |
| Librarianship | — | 2 | — |
| Non science-based occupations | 10 | 5 | — |
| Retiral thro' marriage | — | | 1 |
| Untraced | 67 | — | — |

The B.Sc. Pharmacology Course in Dundee was one of the first to be established, the first student graduating from the Honours Course in 1954. This paper surveys the career patterns of the 197 students who have graduated from this department between 1954 and 1975. Of these, 181 and 53 completed the ordinary and honours B.Sc. courses respectively and 25 students have taken post-graduate degrees in this department (Table 1). All of the honours graduates had previously taken the ordinary B.Sc. course and, of the 25 post-graduates, 9 were recruited from our own honours graduates. The pattern of honours grades awarded showed an even distribution, with the majority of graduates within the upper and lower second class groups.

As is evident from Table 1, 67 of the ordinary B.Sc. graduates were untraced and of the remainder just under half proceeded to honours pharmacology. Thirteen students went directly at this stage into the pharmaceutical industry. Of our 53 honours graduates, approximately half proceeded to further post-graduate study, the other major careers undertaken being medicine and the pharmaceutical industry. Of the 25 students who had taken post-graduate degrees, 15 went into university posts either in teaching or in research and 8 joined the pharmaceutical industry.

In summary, of a total of 133 students followed up (117 Dundee undergraduates plus 16 post-graduates from elsewhere), 64 have entered occupations in which Pharmacology is predominant, 46 have entered careers with a general scientific basis and 20 are known to have entered other occupations, with no scientific basis.

The authors are grateful to the University of Dundee Careers and Appointments Service for information on careers taken up by ordinary B.Sc. graduates.

# DEMONSTRATIONS

## The polymorph transfer reaction: a model system for the study of acute inflammation

J.C. FIRRELL, M.J. PECK & T.J. WILLIAMS
(introduced by G.P. LEWIS)

*Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London, WC2A 3PN.*

An important feature of acute inflammation is an influx of polymorphonuclear leucocytes (PMN-leucocytes). Animal models of acute inflammation (excluding anaphylactic reactions) generally employ local injections of agents such as carrageenin, kaolin or turpentine. In other experiments, immunological responses such as the Arthus reaction, are utilized. These reactions consist of a complex series of events involving mast cells, platelets, complement activation, and leucocyte chemotaxis, etc. As an alternative approach, we have attempted to produce a simplified model system to analyse some aspects of the pharmacology of PMN-leucocytes *in vivo*. The objective of this work was to try to correlate the production of prostaglandins by PMN-leucocytes *in vitro* (Higgs & Youlten, 1972), with the finding that exogenous prostaglandins can potentiate inflammatory exudation *in vivo* (Williams & Morley, 1973).

**Table 1**

| *Treatments (doses/0.1 ml) (n=6)* *1st injection* | *2nd injection* | *Plasma Exudation ± s.e. mean (µl total–14.2* µl control) | *Increased Blood flow ± s.e. mean (% control)* |
|---|---|---|---|
| PMNs ($5 \times 10^6$) | Saline | 10.3 ± 2.3 | 35.6 ± 8.4 |
| Hanks | Bradykinin (0.5 µg) | 63.5 ± 10.3 | 38.3 ± 11.0 |
| PMNs ($5 \times 10^6$) | Bradykinin (0.5 µg) | 123.1 ± 15.9 | 76.4 ± 9.0 |
| PMNs=Indomethacin (1 µg) | Bradykinin (0.5 µg) | 64.9 ± 12.4 | 25.1 ± 10.8 |
| Hanks (control) | Saline | 0.0 ± 1.9 | 0.0 ± 8.5 |

New Zealand White rabbit PMN-leucocytes were prepared from glycogen-induced peritoneal exudate (Hirsch & Church, 1960), and injected intradermally into up to 36 sites on the back of a recipient rabbit ($5 \times 10^6$ PMN-leucocytes in 0.1 ml Hanks solution/site). Control sites received Hanks solution alone. After a fixed interval (60 min in the present experiments), the rabbit was given intravenous $^{131}$I-labelled albumin, and each skin site received a supra-injection of bradykinin mixed with $^{133}$Xe, or $^{133}$Xe in saline alone. Exudation and increased blood flow were measured over the next 30 min as previously described (Williams, 1976a).

Using this technique (which we have termed the 'polymorph transfer reaction'), we have observed clear potentiation of bradykinin-induced exudation in sites injected with PMN-leucocytes. No potentiation of bradykinin responses was observed in sites injected with cells mixed with indomethacin (1 µg/site). The concomitant changes in blood flow were consistent with the results of experiments using exogenous prostaglandins as previously reported (Williams, 1976b).

These results suggest that responses to exogenous bradykinin can be potentiated by endogenous prostaglandin.

This work is supported by the M.R.C.

### References

HIGGS, G.A. & YOULTEN, L.J.F. (1972). Prostaglandin production by rabbit peritoneal polymorphonuclear leukocytes *in vitro*. *Br. J. Pharmac.*, **44**, 330P.

HIRSCH, J.G. & CHURCH, A.B. (1960). Studies of phagocytosis of Group A streptococci by polymorphonuclear leucoytes *in vitro*. *J. exp. Med.*, **111**, 309–322.

WILLIAMS, T.J. (1976a). Simultaneous measurement of local plasma exudation and blood flow changes induced by intradermal injection of vasoactive substances, using [$^{131}$I]-albumin and $^{133}$Xe. *J. Physiol. (Lond.)* **254**, 4–5P.

WILLIAMS, T.J. (1976b). The pro-inflammatory activity of E-, A-, D- and F-type prostaglandins and analogues 16, 16-dimethyl-PGE$_2$ and (15S)-15-methyl-PGE$_2$ in rabbit skin; the relationship between potentiation of plasma exudation and local blood flow changes. *Br. J. Pharmac.*, **56**, 341–342P.

WILLIAMS, T.J. & MORLEY, J. (1973). Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature (Lond.)*, **246**, 215–217.

## Arachidonic acid-induced paw oedema in the rat

J. COTTNEY, A.J. LEWIS &
D.J. NELSON

*Scientific Development Group, Organon Laboratories Limited, Newhouse, Lanarkshire, ML1 5SH*

Arachidonic acid, a prostaglandin percursor, has previously been demonstrated to produce a small but significant, inflammatory oedema when administered by subplantar injection into the hind paw of the rat (Bekemeier, Giessler & Hirschelmann, 1974; Lewis, Nelson & Sugrue, 1975). In addition, when administered into the rat hindpaw in combination with carrageenan or kaolin, arachidonic acid is capable of potentiating the effects of these irritants by a mechanism thought to require conversion of the arachidonic acid to prostaglandins (Lewis *et al.*, 1975).

We have further examined the response produced in the rat hind paw by subplantar administration of arachidonic acid alone in an attempt to establish whether the resulting oedema is a consequence of the conversion of arachidonic acid to prostaglandins which are themselves pro-inflammatory (Glenn, Bowman & Rohloff, 1972).

Male Wistar rats (CE/CFHB, Carworth Europe) weighing between 80–100 g were used in groups of ten for these experiments. Arachidonic acid (Sigma) was dissolved in absolute ethanol before dilution with 0.9% w/v sodium chloride solution and was injected via the subplantar route to the left hind paw. The contralateral paw received an equal volume (0.1 ml) of vehicle. The paw diameters were measured by means of a paw meter (Bonta & Noordhoek, 1973) at regular intervals and paw swelling was calculated as the difference in paw diameters of the archidonic acid treated paw and the control paw. Arachidonic acid (10–250 µg/paw) produced a rapid, dose related swelling maximal between 15 and 30 min (40–50% increase for 100 µg/paw at 15 min) which persisted for as long as 6 hours.

The prostaglandin synthetase inhibitors, indomethacin (1–10 mg/kg) and phenylbutazone (10–100 mg/kg) did not significantly influence the early phase (0–1 h) of this swelling when administered orally in 5% mulgofen 60 min before the injection of 100 µg arachidonic acid. Indomethacin, but not phenylbutazone, partly suppressed the oedema between 1.5 and 3 hours. Major conversion of arachidonic acid to the prostaglandins is therefore unlikely to be responsible for the paw oedema response. An examination of the role played by other likely mediators in this response was then undertaken.

Neither of the histamine antagonists, mepyramine (2.5 mg/kg) and triproline (50 mg/kg) nor the 5-hydroxytryptamine (5-HT) antagonist, methysergide (2.5 mg/kg s.c.) 30 min prior to the injection of arachidonic acid, affected the course of the paw oedema. However, a combination of triprolidine (50 mg/kg) and methysergide (2.5 mg/kg) administered s.c. 30 min prior to arachidonic acid significantly suppressed the early stages of this oedema (0–2 h) This suggests that histamine and 5-HT are released during these early phases of the inflammation.

Pretreatment with either ellagic acid ($2 \times 10^{-4}$ M administered i.v. at 30, 25 and 20 min prior to arachidonic acid) or soybean trypsin inhibitor (SBTI; 80 mg/kg administered i.p. at 4, 2 and 0 h prior to arachidonic acid) both reduced the oedema from 0 to 4 hours. Both ellagic acid and SBTI have been reported to reduce kinin production, ellagic acid by depleting kininogen stores (Gautvik & Rugstad, 1967) and SBTI, by inhibiting kinin formation from kininogen (Maling, Webster, Williams, Saul & Anderson, 1974). Thus arachidonic acid-induced oedema also appears to involve kinin formation.

The combination of ellagic acid and triproline and methysergide did not completely suppress the arachidonic acid oedema, however, and consequently other factors in addition to histamine, 5-HT and kinins, would appear to be implicated in the inflammatory oedema produced by this fatty acid.

### References

BEKEMEIER, H , GIESSLER, A.J. & HIRSCHELMANN, R. (1974). Prostaglandins and inflammation. *Pol. J. Pharmacol.*, **26**, 5–23.

BONTA, I.L. & NOORDHOEK, J. (1973). Anti-inflammatory mechanism in inflamed tissue factor. *Agents Actions*, **3**, 348–356.

GAUTVIK, K.M. & RUGSTAD, H.E. (1967). Kinin formation and kininogen depletion in rats after intravenous injection of ellagic acid. *Br. J. Pharmac. Chemother.*, **31**, 390–400.

GLENN, E.M., BOWMAN, B.J. & ROHLOFF, N.A. (1972). Proinflammatory effects of certain prostaglandins. In *Prostaglandins in Cellular Biology*, ed., Ramwell, P.W. & Pharriss, B.B., pp. 329–344. New York: Plenum Press.

LEWIS, A.J., NELSON, D.J. & SUGRUE, M.F. (1975). On the ability of prostaglandin $E_1$ and arachidonic acid to modulate experimentally induced oedema in the rat paw. *Br. J. Pharmac.*, **55**, 51–56.

MALING, H.M., WEBSTER, M.E., WILLIAMS, M.A., SAUL, W. & ANDERSON, W. (1974). Inflammation induced by histamine, 5-hydroxytryptamine, bradykinin and compound 48/80 in the rat; antagonists and mechanisms of action. *J. Pharmac. exp. Ther.*, **191**, 300–310.

## Class experiment with student volunteers illustrating the influence of formulation on absorption of aspirin

D.K. LUSCOMBE & P.J. NICHOLLS

*Section of Applied Pharmacology, Welsh School of Pharmacy, UWIST, Cardiff, CF1 3NU.*

The influence of formulation on the absorption of drugs is an important aspect of clinical pharmacology. However, such a topic is not readily demonstrated to students since undergraduate practical classes generally operate with constraints such as limited duration of laboratory period, lack of sophisticated equipment and varying technical ability of the students themselves. Nevertheless, we have devised a number of volunteer experiments which can be performed satisfactorily within such constraints and one of these is described below. It is simple to carry out and requires a minimum of practical expertise and equipment. The experiment demonstrates the influence of formulation on the absorption of aspirin using urinary excretion of salicylate over a 2 h period as the index of absorption.

Three different tablet formulations each containing 325 mg aspirin have been employed. Each healthy student volunteer swallows two tablets of one of the formulations with water (150 ml), at least 2 h after a light meal. The bladder is emptied immediately prior to dosing and at 0.5, 1.0, 1.5 and 2.0 hours. The volume of urine at each time is measured and, after alkaline hydrolysis, salicylate content determined colorimetrically using Trinder's reagent (Staff of Department of Pharmacology 1970). Students with a history of gastric disorders, known sensitivity to aspirin or who have not previously taken aspirin, are excluded from the experiment.

With the following fomulations: (A) aspirin with 10% wheat starch (10 s disintegration time, by standard B.P. 1973 test), (B) aspirin with 2% wheat starch (60 min disintegration time) and (C) enteric coated aspirin tablets B.P., typical results (accumulative totals of urinary salicylate, mg mean ± s.e. at 0.5, 1.0, 1.5 and 2.0 h) for groups of eight students are, for A, 5.2 ± 3.0, 15.6 ± 6.3, 25.5 ± 6.8, 39.1 ± 5.4; for B, 0.7 ± 0.2, 2.6 ± 0.7, 6.5 ± 1.2, 12.1 ± 2.4; for C, zero, zero, 0.8 ± 0.3 and 2.1 ± 0.9.

This basic study may be modified to investigate the influence of a variety of orally administered medicines such as antacids, kaolin, iron preparations and antispasmodics on the absorption of salicylate.

We are grateful to Dr N.A. Armstrong for supplying the aspirin tablets.

### Reference

STAFF OF THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF EDINBURGH (1970). Pharmacological experiment on Intact preparations, p. 103, Edinburgh: Livingstone.

---

## Intersubject variability of sulphadimidine acetylation in student volunteers

D.K. LISCOMBE & P.J. NICHOLLS

*Section of Applied Pharmacology, Welsh School of Pharmacy, UWIST, Cardiff, CF1 3NU.*

An acetylation polymorphism affects the metabolism of several drugs such as isoniazid and sulphadimidine. Rao, Mitchison, Nair, Prema & Tripathy (1970) have proposed that classification of individuals into slow and rapid inactivators of isoniazid may be made on the basis of the degree of acetylation of a test dose of sulphadimidine.

We have modified the procedure of Rao *et al.* (1970) for teaching purposes to illustrate the intersubject variation in the acetylation of this sulphonamide. The drug is taken by healthy student volunteers at least 2 h after a light meal and urine is collected between 1 and 3 h after dosing for determination of sulphonamide.

The bladder is voided 1 h before the practical class, a sample of the urine (control) being retained for assay. Each volunteer then swallows four tablets of sulphadimidine B.P. (2 g) with water (150 ml). One hour later, at the commencement of the class, the bladder is again voided, the urine being discarded and a further 150 ml water taken. All urine passed between 1 and 3 h after dosing is collected in a combined volume and assayed colorimetrically for free and total sulphonamide by the method of Bratton & Marshall (1939). Using absorbance values (corrected for control readings) only, the proportion of acetylated sulphadimidine in the urine sample is calculated. This makes it unnecessary to measure volume of urine collected or to run standards through the assay procedure. It is important to thoroughly mix the reagents at each step and to use the coupling reagent freshly prepared. Urine samples seeded with sulphadimidine and its acetylated conjugate are made available for 'practice runs' during the early part of the practical class.

A bimodal distribution for the acetylation of this sulphonamide is evident from the results of the class experiment. Thus, in a group of 57 students the frequency distribution pattern was 10 (0–9% acetylation), 3 (10–19%), 4 (20–29%), 0 (30–39%), 5 (40–49%), 14 (50–59%), 11 (60–69%), 5 (70–79%), 5 (80–89%) and 0 (90–100%). Data obtained from class experiments over the past three years has indicated that even with small groups (i.e. 22 students) this bimodal distribution pattern is obtained. On the basis of these results it would appear that the good acetylators are above the 40% value whereas the poor acetylators are below this figure. If large enough numbers of students participate it may be possible to analyse the data in terms of ethnic groupings.

During the class it is also possible to examine urine samples for the presence of free and acetylated sulphadimidine by thin layer chromatography using silica gel plates containing a fluorescent indicator (Merck F254). Control and test urines (2–3 μl) together with the two pure sulphonamides are applied as separate spots to the plate which is then developed in benzene: acetone (3:1, v/v) for 20 minutes. Sulphadimidine and its acetylated metabolite are then detected as quenching spots (under light at 254 nm) with Rf values of 0.42 and 0.23 respectively.

**References**

BRATTON, A.C. & MARSHALL, E.M. (1939). A new coupling component for sulphanilamide determination. *J. biol. Chem.*, **128**, 537–550.

RAO, K.V.N., MITCHISON, D.A., NAIR, N.G.K., PREMA, K. & TRIPATHY, S.P. (1970). Sulphadimidine acetylation test for classification of patients as slow or rapid inactivators of isoniazid. *Br. med. J.*, **3**, 495–497.

---

## Films in pharmacology teaching

J.R.S. HOULT

*Department of Pharmacology, King's College, London, WC2R 2LS.*

Film loops and full length films, especially those in colour, are used in pharmacology courses to supplement lectures, practicals, and other audio-visual material. Selected film material may thereby increase the scope and interest of courses as well as adding variety to their content.

The film loop, lasting from 2 to 15 min, may be projected on to a conventional screen or displayed on small portable desk-top monitors, and may thus be shown to small groups or to whole classes. It is particularly useful for the demonstration of simple practical techniques, as it can be allowed to run and repeat itself until the student is familiar with the experimental technique. The use of close-up and colour enables the clear visualization of detail which may not always be possible during live demonstrations. In addition, film loops provide economies in demonstrator time and permit students to learn independently at their own rate. The production of film loops is well within the capabilities and budget of many university audio-visual departments.

The expense and time involved in making a first class full-length film puts film-making outside the scope of all but the largest organizations. However, there are many excellent films on pharmacological topics which are available from the pharmaceutical industry and from other sources. In many cases these are not orientated towards commercial promotion, and may be hired free or at nominal cost. Such films cover many aspects of drug actions and their therapeutic uses, as well as relevant physiological and clinical topics. Obvious benefits of the use of films include the convenient access to material which would otherwise be unavailable to the student, and the juxtaposition of basic theory with experimental and clinical material. Imaginative use of animated diagrams, colour, cinemicrophotography, slow motion sequences, and other techniques, contribute to the powerful impact and teaching effectiveness of films.

The intention of this demonstration is (a) to show examples of films and film loops which illustrate some of the points discussed above, and which demonstrate the potential value of films in pharmacology teaching programmes; and (b) to provide catalogued information and basic details about film material which is now available, both from academic and commercial sources. Much of the information concerning films made by pharmaceutical companies has been obtained from a recent questionnaire (April–May 1976) which shows that many companies have a lively interest in pharmacology teaching.

I wish to acknowledge the kind co-operation of many pharmaceutical companies in providing information, and especially to those who have generously loaned films for this demonstration.

## Effective pharmacological demonstrations to 100 students

A. D'MELLO & Z.L. KRUK

*Department of Pharmacology and Therapeutics, The London Hospital Medical College, London, E1 2AD.*

Pharmacology is a practical science and its teaching is greatly assisted by experiments which demonstrate and reinforce well known basic principles and actions of drugs. Efficient teaching of practical pharmacology to 100 students is difficult to organize, requires large laboratories, large amounts of apparatus, and inevitably requires a large number of staff.

As part of a comprehensive course in practical pharmacology for medical students we demonstrate the effects of drugs on the peripheral nervous system (anaesthetized cat, isolated heart and intestine preparations, neuromuscular junction in skeletal muscle) using a multi-channel recorder with overhead projection facilities ('Physiograph'; Narco Biosystems, Inc., U.S.A.).

The demonstrations are conducted in a conventional tiered lecture theatre with a large projection screen. The advantages of this arrangement are obvious:

- low staff/student ratio (two academic and two technical staff for 100 students)
- minimization of apparatus failure and elimination of poor experimental design
- elimination of lack of students' expertise
- supervised discussion on every aspect of the demonstration with full encouragement given to voice every opinion so that misunderstandings and errors of judgement and fact can be rectified.

The students are encouraged to make full notes during the demonstration and discussions, and are required to present a concise account of events. To help them a statement on the main aims and objectives of the demonstration is available, as are specimen traces of results.

We have tried to assess the effectiveness of this method of teaching by comparing the performance of students in determinate multiple choice questions on aspects of peripheral nervous system pharmacology which had been demonstrated and other aspects which had not been demonstrated. We were encouraged by the following results obtained in two successive years (1974, 1975). The mean facility ± s.e. mean (defined as the percentage of students making the correct response) for demonstrated aspects ($85 \pm 2.9$ in 1974; $85 \pm 2.6$ in 1975) was significantly greater ($P < 0.01$) than the mean facility for non-demonstrated aspects ($70 \pm 3.3$ in 1974; $68 \pm 5.2$ in 1975).

To give the students an idea of their own performance and to help us to identify problem areas in knowledge and understanding, we have introduced short (about 30 items) determinate tests at the end of a series of demonstrations. The students mark each others' answer sheets and calculate the score. Each individual item is then assessed for facility, and any problem questions (we take a facility of $<60$ as the cut off point) is discussed again in the hope of correcting misconceptions or supplying facts where there were areas of ignorance.

We consider that this method has many advantages over conventional large practical classes especially in these times of increasing student numbers and restriction on appointment of new staff. There has been no decrease in standards and from our experience it is popular with both staff and students.

---

## Developments in the use of television for pharmacology teaching

G.D.H. LEACH

*University of Bradford.*

Since the earlier demonstration given to the Edinburgh meeting of the Society in 1974, two developments of practical interest to pharmacologists could be noted:

(i) Colour productions.

In these cases, original monochrome productions have been updated and the use of all colour or partial colour television material has been introduced into the teaching curriculum. In the tapes to be demonstrated, different aspects of the usefulness of such material will be emphasized.

(a) An all colour presentation of the recording of nerve action potentials in the anaesthetized animal will be shown. The tape has been prepared in the studios of the University of Aberdeen and is presented by Professor Malcolm.

(b) A series of partial colour productions prepared by Dr Foy, Dr McCurrie and Professor Leach of the University of Bradford will also be shown.
These will include relevant aspects of lecture demonstration material from areas such as mammalian blood pressure and respiration recording, mechanism of muscle relaxant action and the actions of sympathomimetics on the Langendorff perfused heart preparation.

## Slide-tape programmes in the teaching of pharmacology

B. COX, J.R. FOZARD &
R.C. SMALL

*Department of Pharmacology, Materia Medica and Therapeutics, University of Manchester, Manchester, M13 9PT.*

A series of slide-tape teaching programmes has been developed to aid the teaching of pharmacology at Manchester (Table 1). Principally these programmes are intended for private study by individual students. They are expected to be used

(a) by students (undergraduate and postgraduate) wishing to pursue private revision.
(b) as revision courses for students experiencing difficulty with more traditional forms of teaching.
(c) by postgraduate students following M.Sc. courses in Pharmacology after first degree courses in related subjects and for foreign postgraduate students.
(d) in some cases by one particular sub-group of students (e.g. dental students) as the main part of their course.[1]

The demonstration will show selected samples from the slide-tape library including programmes on local hormones and mediators on inflammation, local anaesthetics and autonomic pharmacology for dental students.

The techniques for rapid and relatively inexpensive production of tapes will be presented along with surveys of student attitudes to this form of teaching. More detailed evaluation is underway.

---

[1] This programme was made in collaboration with the Dental School, University of Los Angeles, California, U.S.A.

**Table 1** Slide-tape programmes available for teaching Pharmacology

| *Programme* | *No. of tapes* | *Topic* | *Audience* |
|---|---|---|---|
| 1 | 1 | Introduction to the autonomic nervous system | General |
| 2 | 3 | The noradrenergic neurone | General/advanced |
| 3 | 3 | Local hormones and mediators of inflammation | General |
| 4 | 3 | Endocrine pharmacology | General |
| 5 | 3 | Pharmacology of reproduction | General/M.Sc. Reproductive Pharmacology |
| 6 | 1 | Pharmacology of the eye | General/Ophthalmic optics |
| 7 | 1 | Local Anaesthetics | |
| 8 | 1 | Drug Metabolism | General |
| 9 | 1 | Malaria | General |
| 10 | 8 | Autonomic Pharmacology | Dentists |

## Autonomic drugs and the eye

J.M. FOY & D.J. HOPKINS

*School of Pharmacology, University of Bradford, Bradford, BD7 1DP and Department of Ophthalmology, Bradford, BD9 6RJ.*

This tape-slide presentation takes the form of a discussion between a pharmacologist and a consultant ophthalmic surgeon and centres on the latter's choice and use of drugs active on the eye.

The tape opens with some revision material on the structure, function and innervation of the eye followed by a classification of the different ways of drug administration. There is then a categorization of the different autonomic agents to be discussed later. An extended section on glaucoma, both acute and chronic, reflects the surgeon's interest in this disease and clarifies for the non-specialist the important differences between the two forms. The remaining discussion on drugs and their use in ophthalmology is somewhat less detailed but nevertheless contains many interesting photographs taken in clinical situations. The inclusion of drugs in the presentation is not meant to be comprehensive but is restricted to those actually used by the clinician.

## Teaching methods in pharmacology

J.M. FOY & JUDITH SENIOR

*School of Pharmacology, University of Bradford, Bradford, BD7 1DP*

A short videotaped recording where final year students comment on the various teaching methods in which they have been involved.

---

## *Langendorff* mammalian heart perfusion

G.D.H. LEACH

*School of Pharmacology, University of Bradford, Bradford, BD7 1DP*

A colour, videotaped recording showing the effect of some common drugs on the isolated perfused rabbit heart.

---

## The blood pressure and respiration of the anaesthetized cat

J.M. FOY & J.R. McCURRIE

*School of Pharmacology, University of Bradford, Bradford, BD7 1DP.*

This colour, videotaped recording is designed as an initial demonstration to be shown towards the end of the first year in an undergraduate course. It contains demonstrations, recorded during actual experiments, of some important features of the control of blood pressure and respiration.

# A new method of obtaining human skin inflammatory exudate for pharmacological analysis

A. KOBZA BLACK, M.W. GREAVES, C.N. HENSBY & N.A. PLUMMER

*Institute of Dermatology, St John's Hospital for Diseases of the Skin, Homerton Grove, London, E9 6BX.*

The value of the perfusion technique in obtaining inflammatory exudate is limited by its relative insensitivity and lack of quantification, due to the diluting effect of the infusing fluid (Greaves & Søndergaard, 1971).

In order to overcome these problems we have devised a new method in which fluid contents of suction bullae raised on inflamed skin are used as a source of exudate. Bullae, 6 mm in diameter were raised at a pressure 200 mmHg below atmospheric, in approximately 120 minutes. Volumes of exudate of 0.5 ml or more were aspirated immediately into a polythene syringe with a 26 gauge needle. Exudates from skin inflamed by topical application of tetrahydrafurfuryl nicotinate (Thurfyl nicotinate) and from normal skin were compared. Thurfyl nicotinate exudates tested on a superfusion cascade bioassay system caused contractions of the rat stomach and rat colon equivalent to 48.3 ± 7.5 ng/ml, $n = 15$ (mean ± s.e. mean) of prostaglandin (PG)-like activity estimated in $PGE_2$ equivalents. Exudate from normal skin showed 14.6 ± 4.4 ng/ml ($n = 18$) $PGE_2$ equivalents. There was no increase shown either in histamine or in bradykinin in the thurfyl nicotinate inflamed skin.

The presence of significantly increased $PGE_2$ and $PGF_2$ activity in the thurfyl nicotinate reactions was subsequently confirmed by gel partition chromatography and radio-immunoassay. Prior oral administration of aspirin (600 mg 4 hourly for 24 h) decreased PG-like activity, and reduced the thurfyl nicotinate erythema.

Light and electron microscopic studies revealed the level of separation of the bulla to be in the septa lucida, between the basal cell plasma membrane and the basal lamina. Other physical characteristics of the exudate from normal and thurfyl nicotinate inflamed skin will be demonstrated.

## References

GREAVES, M.W. & SØNDERGAARD, J. (1971). Continuous skin perfusion *in vivo* as a method for study of pharmacological agents in human skin. *Acta Dermatovenor (Stockholm)*, **51**, 50–54.

---

# Aberdeen-Dundee medicines evaluation and monitoring group

R.D. WIER, D.C. MOIR, Z.G. ERSKINE, S.C. GALLON, J.F. DAVIDSON, J. CROOKS & L.J. CHRISTOPHER

*Department of Community Health, University of Aberdeen and Department of Pharmacology and Therapeutics, University of Dundee.*

The Aberdeen-Dundee Medicines Evaluation and Monitoring Group (MEMO) has evolved from a drug monitoring system developed in Aberdeen since 1964. The main activities of the group include investigations into:

(i) suspected drug adverse reaction associations,
(ii) aspects of drug efficacy,
(iii) drug utilization, and
(iv) an educational role related to maintenance of the hospital drug handling system and feedback of information derived from the above.

The data base of the system is the linkage of individual patient records of drug presentation and administration, to hospital morbidity data. A computer file of over one million prescriptions and 250,000 discharges has thus been created indexed by age, sex, diagnosis or drug, as required.

The staff comprising in whole-time equivalents — 4 professional workers and 5$\frac{1}{4}$ clerical assistants now handle 70,000 discharges and 250,000 prescriptions per year from a total population of 750,000.

The basic system is presented and the following topics selected to illustrate different aspects of its activities: (a) processing drug names, (b) improving hospital prescribing, (c) information for users, (d) prescribing outwith hospital.

# Studies on *Azadiracta indica* in malaria

A. TELLA

*Department of Pharmacology, College of Medicine of the University of Lagos.*

Primary acute blood-transmitted *P. berghei* malaria was induced in young male albino mice (Tella & Maegraith, 1965) divided into three groups. One group represented the controls in which the infection was not treated. The second group was treated with daily oral doses of the leaf extract of *Azadiracta indica*. The third group received dapsone.

In the first two groups of animals, reticulocytosis did not occur, but the parasite count rose, packed cell volume and erythrocyte counts fell with each day of the infection which ended fatally on the fifth day. The third group recovered completely indicating that the infection, although acute, was still capable of responding favourably to suitable drugs.

The results thus show that *Azadiracta indica* leaf is ineffective in *P. berghei* malaria contrary to the claim that it is of therapeutic value in human malaria.

## References

TELLA, A. & MAEGRAITH, B.G. (1965). Physiopathological changes in primary acute blood-transmitted malaria and babesia infections. *Ann. Trop. Med. Parasit.*, **59**, 135–146.

# INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac,* **50**, 3-23, may also be obtained. Specific points to note are:

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors **in alphabetical order.** A short title of not more than 50 letters should also be suggested.

2. **Statement** Papers are accepted only if accompanied by a statement that they have not been and will not be published in whole or in part in any other Journal.

3. **Summary** (2 copies) This should be a short summary of results and conclusions arranged in numbered paragraphs.

4. **Abstract** (2 copies) An abstract of about 100 words suitable for inclusion in an abstracting journal should be typed on a separate sheet, giving the author's name and address and the title of the paper.

5. **Figures** These should not be larger than A4 (206 x 294 mm) and may be in the form of original drawings, recorded tracings or high quality photographic prints made from them. Negative prints of kymograph tracings (black on white) should be provided, and both ordinates and abscissae should be calibrated. Lettering on figures should be inserted in pencil. Symbols which are to appear in figures or legends should be chosen from the following:

○ ● □ ■ △ ▲ ▽ ▼

Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction.

6. **Tables** These should not have more than 85 characters to a line (counting spaces between columns as 4 characters) and certainly not more than 110 characters to a line, unless absolutely unavoidable. **Figure legends** and **tables** should be typed on separate sheets of paper.

7. **References** These should be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the fourth edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should in addition, include the names of editors, the edition number, where appropriate, and the town of origin and name of publisher.

**Two page proofs** will be supplied, one of which may be retained by the authors. The other should be corrected immediately and returned to the Press Editor. Corrections should be kept to a minimum.

**Twenty-five reprints** will be supplied to the authors free of charge. Additional reprints can be purchased. A reprint order form, which should be returned promptly, will be sent out with the proofs. No reprints of abstracts are supplied.

**Short Communications** These may be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text and only one figure or table. The summary should be a single paragraph. An abstract and statement should be included.

# British Journal of Pharmacology

VOLUME 58 NUMBER 2 OCTOBER 1976

165 COLINA-CHOURIO, J., McGIFF, J.C., MILLER, M.P. & NASJLETTI, A. Possible influence of intrarenal generation of kinins on prostaglandin release from the rabbit perfused kidney

173 BRIATICO, G., GUISO, G., JORI, A. & RAVAZZANI, C. Effects of contraceptive agents on drug metabolism in various animal species

183 STEINESS, EVA & VALENTIN, N. Myocardial digoxin uptake: dissociation between digitalis-induced inotropism and myocardial loss of potassium

189 KISIN, I.E. On the decrease of myocardial oxygen consumption induced by chloracyzine

193 COLLIER, H.O.J., McDONALD-GIBSON, WENDY J. & SAEED, S.A. Stimulation of prostaglandin biosynthesis by drugs: effects *in vitro* of some drugs affecting gut function

201 FERREIRA, S.H. & SMAJE, L.H. Bradykinin and functional vasodilatation in the salivary gland

211 APPERLEY, EIRA, HUMPHREY, P.P.A. & LEVY, G.P. Receptors for 5-hydroxytryptamine and noradrenaline in rabbit isolated ear artery and aorta

223 ABOULAFIA, JEANNINE, MENDES, GUN B., MIYAMOTO, MARIA E., PAIVA, A.C.M. & PAIVA, THEREZINHA B. Effect of indomethacin and prostaglandin on the smooth muscle contracting activity of angiotensin and other agonists

229 DALE, M. MAUREEN, EVINÇ, AKGÜN & VINE, PAULINE. The denervated cremaster muscle of the guinea-pig as a pharmacological preparation

239 BEVAN, P., BRADSHAW, C.M. & SZABADI, E. The action of micro-electrophoretically applied L-3,4-dihydroxyphenylalanine (DOPA) on single cortical neurones

247 CLANACHAN, A.S. & McGRATH, J.C. Effects of ketamine on the peripheral autonomic nervous system of the rat

253 SMITH, G.W. & SOMOVA, LILIANA I. Renal function and renal venous prostaglandin concentrations during different stages of experimental renal hypertension in the rat

261 KALSNER, S. The lack of effect of oxytetracycline on responses to sympathetic nerve stimulation and catecholamines in vascular tissue

267P PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY, Dundee, 15–16 July, 1976

Printed in Great Britain by The Whitefriars Press Ltd., London and Tonbridge

BRITISH HIGH COMMISSION,
BRITISH COUNCIL DIVISION,
5, Shakespeare Sarani,
Calcutta-700016

# British Journal of Pharmacology

November 1976

NOT TO BE LENT OUT

24

# Edited for the British Pharmacological Society by

A.W. Cuthbert *(Chairman)* H.M. Adam *(Secretary)*
Margaret Day *(Press Editor)*
J.D. Stephenson *(Assistant Press Editor)*

A.M. Barrett
W.G. Bisset
T.B. Bolton
D.A. Brown
B.A. Callingham
T.J. Crow
F. de Matteis
C.T. Dollery
V. Eisen
E.W. Gill
A.F. Green
D.F. Hawkins
Margarethe Holzbauer
D.B. Hope
J.S. Kelly
G.M. Lees
G.P. Lewis
I.H.M. Main
E.K. Matthews
J.F. Mitchell
P.A. Nasmyth
M.W. Parkes
M. Sandler
A.P. Silverman
M.A. Simmonds
F.M. Sullivan
A. Ungar
R.A. Webster

---

Papers will be considered for publication on all aspects of drug action, including chemotherapy.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ. Authors should consult the instructions inside the back cover.

*The British Journal of Pharmacology* is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription £45.00 (USA & Canada $99. Rest of world $92). (Orders must be accompanied by remittance. Prices correct at time of publication.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

Enquiries concerning advertising space and rates should be addressed to: Advertisement Department, Macmillan Journals Limited, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Copyright © 1976 by the *British Journal of Pharmacology*. All rights of reproduction are reserved in respect of all papers, articles, illustrations, etc., published in this journal in all countries of the world.

Second class postage paid at New York, N.Y.
US Mailing Agent, Expeditors of the Printed Word, Ltd,
527 Madison Avenue, New York, NY 10022.

# British Journal of Clinical Pharmacology

**THE BRITISH JOURNAL OF CLINICAL PHARMACOLOGY** provides a forum for the exchange of information and ideas between the pharmaceutical industry and the medical profession. First published just over two years ago, the BJCP has in this very short time become the most authoritative channel of communication between these two areas of research and practice in clinical pharmacology. By presenting the latest findings of new research, while keeping the ultimate object of patient care always in mind, the BJCP acts as a vitally needed educator in this fast-moving area of science.

BJCP contains papers and reports on all aspects of drug action in man, in both health and in disease. These include the pre-clinical testing of drugs in normal volunteers and important clinical trials in patients. Discussions often clarify the relationships between different drug therapies, and between drug therapies and non-drug therapies; the latter tend to be rather neglected in therapeutics. Particular attention is paid to advances in techniques, the methodology of study and investigations of more scrupulous clinical practice.

The editors contribute informed comment and evaluation of papers in terms of contemporary clinical practice, while letters and short communications allow immediate discussion of urgent observations and negative reports.

**THE BRITISH JOURNAL OF CLINICAL PHARMACOLOGY** is as fast-moving as the area of science it examines. Its expanding international readership testifies to its effectiveness as a vehicle of information and communication. Why not join the ever growing number of clinical pharmacologists all over the world who rely on the BJCP as their professional journal?

---

## Subscription Order Form

To: Subscription Dept., Macmillan Journals,
Brunel Road, Basingstoke RG21 2XS, Hampshire, England.
Please enter me for a year subscription to the
**British Journal of Clinical Pharmacology.**
**Annual Subscription £28·00 (U.S.A. and Canada U.S.$65, Rest of World U.S.$60)**
(Payment must accompany order; cheques should be made payable to Macmillan Journals Limited; payment may be made in any currency at the current exchange rate.)

I enclose..........................................................................................................

Name..............................................................................................................

Address...........................................................................................................

.......................................................................................................................

**Prices correct at time of publication**
**Registered No. 785998. Registered Office: 4 Little Essex Street, London WC2R 3LF.**

# A Symposium

*will be held under the auspices of the*

**Physiological Society**

*at King's College, Strand, London WC2R 2LS*
*on Friday, December 10th, 1976*
*on*

**'New Aspects of Epithelial Transport'**

*at which electrolyte and fluid absorption*
*and secretion in mammalian, amphibian and insect*
*epithelia will be discussed.*

---

All interested in attending the Meeting
should contact

**The Secretary,**
**Department of Physiology,**
**King's College, London.**

---

**FITC-DEXTRANS**

**for permeability and microcirculation studies in animals**

**Well characterized tracer substances**

- fluorescein label for easy detection
- ultra-micro determination in nanolitre sample volumes
- narrow molecular weight distribution
- five fractions between 3,000 and 150,000 daltons

FITC-Dextrans and other products for biological research are described in the booklet "Biomedical Research. Product guide", free on request.

Pharmacia (Great Britain) Ltd.
Paramount House,
75 Uxbridge Road,
London W5 5SS

Pharmacia
Fine Chemicals

VOLUME 58(3) NOVEMBER 1976

# British Journal of Pharmacology

323 CLARK, A.L. & MITCHELSON, F. The inhibitory effect of gallamine on muscarinic receptors

333 EAKINS, K.E., RAJADHYAKSHA, V. & SCHROER, R. Prostaglandin antagonism by sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl]phenyl phosphonate (N-0164)

341 SEGAL, M. Glutamate antagonists in rat hippocampus

347 RICHARDS, C.D. & SMAJE, J.C. Anaesthetics depress the sensitivity of cortical neurones to L-glutamate

359 SMAJE, J.C. General anaesthetics and the acetylcholine-sensitivity of cortical neurones

367 KRUK, Z.L. & ZARRINDAST, M.R. Mazindol anorexia is mediated by activation of dopaminergic mechanisms

373 BISCOE, T.J., EVANS, R.H., HEADLEY, P.M., MARTIN, M.R. & WATKINS, J.C. Structure-activity relations of excitatory amino acids on frog and rat spinal neurones

383 DEY, P.K. & FELDBERG, W. Analgesia produced by morphine when acting from the liquor space

395 BARTLET, A.L. Actions of carbon tetrachloride, hexachloroethane and the products of their metabolism in sheep on *Fasciola hepatica*

401 HENNINGSSON, S. & ROSENGREN, ELSA. The effect of nandrolone, an anabolic steroid on putrescine metabolism in the mouse

407P PROCEEDINGS OF THE BRITISH AND FRENCH PHARMACOLOGICAL SOCIETIES, The University of Oxford, 15–17 September, 1976

## PROCEEDINGS OF THE BRITISH AND FRENCH PHARMACOLOGICAL SOCIETIES

The University of Oxford, 15th–17th September, 1976

407P MARSHALL, R.J. & PARRATT, J.R. The effects of dobutamine in the early stages of acute experimental myocardial infarction in the dog

408P DELAIGUE, R., DUCHENE-MARULLAZ, P., GUEORGUIEV, G. & KANTELIP, J.P. Comparative effects of chloralose and pentobarbital on a atrioventricular conduction in the dog

409P FARGES, J.P., FAUCON, G. & OLLAGNIER, M. Heterogeneous reactivity of atrial and ventricular cardiac contractile tissue

409P DUPUIS, B., GAUTIER, P., RICHER, G. & SERRE, M. A method for experimental study of arrhythmias following myocardial infarction in closed-chest dog. Its use as a test for antiarrhythmic drugs

410P GARNIER, D., GOUPIL, N., NARGEOT, J., POURRIAS, B. & ROUGIER, O. Effects of tryptamine on mechanical and electrical activities of sino-atrial frog auricular trabeculae

410P BERDEAUX, A., BOISSIER, J.R. & GIUDICELLI, J.F. Autonomous control of the heart, myocardial blood flow and S-T segment in canine ischaemic myocardium

411P BERDEAUX, A., BOISSIER, J.R. & GIUDICELLI, J.F. Atenolol, regional myocardial blood flow and S-T segment in canine ischaemic myocardium

411P CHARLIER, R.H., RAUTHIER, J.A. & RICHARD, J.C. An indolizine with an amiodarone-like haemodynamic profile

412P TAYLOR, S.H. & THADANI, U. Oxprenolol in angina pectoris

412P HARICHAUX, P. & HARY, L. Some complementary data on AH 5158, an inhibitor of both $\alpha$- and $\beta$-adrenoceptors

413P IMBS, J.L., SCHMIDT, M. & SCHWARTZ, J. Effect of (±)-propranolol on the recovery of urinary concentration process after frusemide, in the rat

413P DE POVER, A. & GODFRAIND, T. The role of potassium in the inhibition by cardiac glycosides of (Na$^+$-K$^+$)-ATPase prepared from human heart

415P HONERJÄGER, P. & REITER, M. (introduced by Trendelenburg, U.). Batrachotoxin: activity-dependent prolongation of the cardiac action potential and positive inotropic effect

416P FOZARD, J.R. & MOBAROK ALI, A.T.M. Inhibition of the stimulant effect of 5-hydroxytryptamine on cardiac sympathetic nerves by 5-hydroxytryptamine and related compounds

417P BOULU, R.G., LARSEN, R.G., PLOTKINE, M. & ROSSIGNOL, P. Effects of carbonic anhydrase inhibitors upon cerebral cortex oxygen availability and resistance to hypoxia

417P BOISMARE, F.R., HACPILLE, L., LEFRANCOIS, J.P. & LEPONCIN, M. Long-lasting effects of the opening of the blood brain barrier on the modifications induced by intracarotid injection of noradrenaline

418P BEVAN, P., BRADSHAW, C.M. & SZABADI, E. Neuronal responses to adrenoceptor agonists in the cerebral cortex: evidence for excitatory $\alpha$-adrenoceptors and inhibitory $\beta$-adrenoceptors

419P DESOIZE, B., JARDILLIER, J.C., LEGER, C. & NAHAS, G.G. Delta-9-tetrahydrocannabinol (THC) and macromolecular synthesis: mechanisms of action

419P PASCAUD, X.B. & ROGER, A.R. Is the gastric antisecretory property of clonidine in rats of central origin?

420P BAKER, G.B., BERTOLLINI, A., del CARMINE, R., MARTIN, I.L. & RAITERI, M. Effects of *p*-chloro-$\beta$-phenylethylamine on the uptake and release of putative amine neurotransmitters in rat brain

422P FLUDDER, JOAN M. & TONGE, SALLY R. (introduced by Leonard, B.E.). Modification by oestrogen of the effects of (D)-amphetamine sulphate on noradrenaline metabolism in discrete areas of rat brain

423P AMBROZI, L., BIRKMAYER, W., RIEDERER, P. & YOUDIM, M.B.H. L-Dopa and (–)-deprenil in the treatment of Parkinson's disease: a long-term study

424P RICHARDS, J.G. Autoradiographic evidence for the effects of specific uptake-inhibitors on the selective accumulation of [$^3$H]-5-HT by supra-ependymal nerve terminals and for the localization of binding sites for [$^3$H]-DLSD

425P FILLION, G., FILLION, M.P., JACOB, J. & ROUSSELLE, J.C. 5-HT and LSD high affinity binding sites to brain synaptosomal membranes

426P HILLIER, K., ROBERTS, P.J. & WOOLLARD, P.M. Catecholamine-stimulated prostaglandin synthesis in rat brain synaptosomes

427P HILEY, C.R. Ontogenesis of muscarinic receptor sites in rat brain

428P COLQUHOUN, D. Analysis of end-plate current fluctuations produced by acetylcholine and acetylmonoethylcholine in rat muscle

429P LOUBATIÈRES, A.L., RIBES, G. & VALETTE, G. Pharmacological agents and acute experimental hyperlactataemia in the dog

430P CHUBB, I.W. & RAINE, A.E.G. (introduced by Vaughan Williams, E.M.). Long-term effects of propranolol on tyrosine hydroxylase and dopamine-$\beta$-hydroxylase in the superior cervical ganglia of the rabbit

431P AUCLAIR, M.C., JOURDON, P., LECHAT, P. & LEMEIGNAN, M. Action of cytochrome C on transmembrane potentials of normal or hypoxic guinea-pig myocardial strips

431P FARRAR, D.G. & VERITY, ANNE. Analysis of the effects of isoxsuprine on guinea-pig atria and trachea

432P ALTMAN, J., Da PONTE, F. & WORCEL, M. Altered reactivity of stomach fundus smooth muscle in Okamoto spontaneous hypertension

433P CHEVILLOTTE, E., DEVYNCK, M.A., MEYER, P., PAPADIMITRIOU, A., ROUZAIRE-DUBOIS, B. & WORCEL, M. Molecular mechanism of post-nephrectomy uterine supersensitivity to angiotensin

434P LUGNIER, C., MICHON, T. & STOCLET, J.C. Evolution of aortic and cardiac cyclic AMP phosphodiesterase during the onset of mineralocorticoid hypertension in the rat

435P COHEN, Y., LINDENBAUM, A., PORQUET, D. & WEPIERRE, J. Evidence of central cardiovascular effects of intracerebroventricular isoprenaline in anaesthetized rat

435P NAHORSKI, S.R. & SMITH, BRENDA M. Characteristics and altered sensitivity of cerebral $\beta$-adrenoceptors assessed by [$^3$H]-propranolol binding

436P DUBOST, M., ESCOUSSE, A., MOUNIÉ, J. & TRUCHOT, R. Peripheral effects of the amphetamine-type anorectic drugs: inhibition of catecholamine-induced lipolysis, respiration, glucose utilization in the adipose tissue of man and rat

437P DUMEUR, G., HÜE, BERNADETTE, LWOFF, J.M., MOURIES, M.A. & TREMBLAY, D. Activity of anorectic drugs (amphetamine), amfepramone and UP 507–04) on two models of obesity in animals

437P MARCY, R. & QUERMONNE, M.A. Decremental skin conductance response in mice, during iterative photostimulation; an attention-sustaining capacity model for psychopharmacological research

439P CLARKE, CORAL H., NICHOLSON, A.N. & STONE, BARBARA M. Effect of the 1,5-benzodiazepines, clobazam and triflubazam, on the sleep of man

439P SILVERMAN, A.P. Behavioural changes in rats suggesting drug-induced headache

440P FAGG, G.E., JORDAN, C.C. & WEBSTER, R.A. The release of endogenous amino acids from the cat spinal cord *in vivo*

441P LEWIS, D.A. & PARROTT, D.P. The effect of cortisol on $\alpha_1$-macroglobulin and $\alpha_2$-acute phase globulin in the arthritic rat

442P MILLER, P. & ROBSON, P. (introduced by James, G.W.L.). Potentiation of anaphylactic bronchoconstriction by non-steroidal anti-inflammatory agents

443P DELBARRE, B., SCHMITT, H. & SENON, DANIELLE. Effects of activation of $H_1$ and $H_2$-receptors on central cardiovascular structures in cats and on behaviour in chickens

444P DUCKWORTH, J.W., LANCE, J.W., LORD, G.D.A. & MYLECHARANE, E.J. (introduced by Raper, C.). Histamine receptors in the cranial circulation of the monkey

445P BURKA, J.F., EYRE, P. & HOLROYDE, M.C. Dopaminergic (co-mediator) modulation of release of histamine and SRS-A in the calf

446P WHITTLE, B.J.R. Calcium and the inhibition of histamine release from rat peritoneal mast cells by non-steroid anti-inflammatory agents

447P VARGAFTIG, B.B. Effects of prostaglandin synthetase inhibitors and of other drugs on aggregation of plasma-free rabbit platelets by carrageenan and by thrombin

448P ADOLPHE, M., DUNN, C.J., FONTAGNÉ, J., GIROUD, J.P., LECHAT, P., PAUBLANT, D. & WILLOUGHBY, D.A. Mitogenic activity of different types of non-immunological inflammatory exudates on macrophages in culture

448P CONOLLY, M.E., GREENACRE, J.K. & SCOFIELD, P. Dose-dependent desensitization of lymphocyte beta-receptor function after long-term culture with isoprenaline

450P ALRIC, R., LIGNON, F., LOUBATIÈRES, A.L., MANTEGHETTI, M. & PUECH, R. Non-saturable co-operative binding of tolbutamide to isolated islets of Langerhans

450P ISMAHAN, G., PARVEZ, H. PARVEZ, S. & RAZA-BUKHARI, A. Interrelations between MAO activity and carbohydrate metabolism

451P DEWS, P.B. Effects of drugs on suppressed responding

452P BOISSIER, J.R., DUMONT, C., OBERLANDER, C. & PETERFALVI, M. A comparison of circling behaviour induced in nitro-striatal lesioned rats after peripheral administration of indole derivatives

453P WADDINGTON, J.L. (introduced by Crow, T.J.). A behavioural model of the GABA-facilitating action of benzodiazepines: rotational behaviour after unilateral intranigral injection of chlordiazepoxide

454P KOOB, G., DEL FIACCO, MARINA & IVERSEN, SUSAN D. The behavioural effects of EOS-induced changes in substantia nigra GABA levels

454P BOISSIER, J.R., GODEFROY, F., SOUBRIE, P., THIEBOT, M.H. & WEIL-FUGAZZA, J. Platelet uptake of [$^{14}$C]-5-hydroxytryptamine in 'emotional' and 'non-emotional' rats

455P CURZON, G. & MARSDEN, C.A. Effects of *p*-chlorophenylalanine and $\alpha$-methyltryptophan on rat social behaviour

456P BOWERY, N.G. Reversal of the action of $\gamma$-aminobutyric acid (GABA) antagonists by barbiturates

458P BREUKER, E., DINGLEDINE, R. & IVERSEN, L.L. Evidence for naloxone and opiates as GABA antagonists

458P DAVIES, J. & DRAY, A. Actions of enkephalin and morphine on spinal cord and brain stem neurones

459P HILL, R.G. & PEPPER, C.M. The effects of morphine and metenkephalin on nociceptive neurones in the rat thalamus

460P BIRDSALL, N.J.M., BRADBURY, A.F., BURGEN, A.S.V., HULME, E.C., SMYTH, D.G. & SNELL, C.R. Interactions of peptides derived from the C-fragment of $\beta$-lipotropin with brain opiate receptors

462P ARMSTRONG, J.M. & LATTIMER, N. Prostaglandin E$_2$ released from the kidneys of genetic hypertensive rats contributes to the vasoconstrictor supersensitivity to angiotensin II *in vitro*

463P CARDINAL, D.C. & HILL, T.W.K. (introduced by Walker, J.M.G.). A device for controlling perfusion pressure

464P DAWES, P. & REDFERN, P.H. A simple and inexpensive method for the intracerebral administration of drug solutions to the conscious rat

464P CLARKE, G. & LINCOLN, D.W. Central and peripheral inhibition of the milk-ejection reflex: studies with $\mu$-adrenoceptor antagonists

465P FILE, SANDRA E., HYDE, J. & POOL, M. (introduced by Neal, M.J.). Effects of ethanol and chlordiazepoxide on social interaction in rats

466P de BONO, D.P., GORDON, J.L., MacINTYRE, D.E. & PEARSON, J.D. Active transport of adenine and adenosine by blood platelets and cultured endothelial cells

467P LEWIS, D.M. & WEBB, SANDRA N. Adrenaline on isotonic contractions of mammalian skeletal muscle

468P KRUK, Z.L., SMITH, L.A. & ZARRINDAST, M.R. Antagonism of responses to anorectics by selective receptor blockers

469P JENNER, P.G. & PYCOCK, C.J. Interaction of clonidine with dopamine-dependent behaviours in rodents

470P PILOT, MARIE-ANNE (introduced by D'Mello, A.). Indomethacin and acid secretion in the isolated perfused canine stomach

471P BOOT, J.R., DAWSON, W. & OSBORNE, D.J. The biological significance of prostaglandin-like substances released from immunologically challenged guinea-pig lungs

472P BOLAM, J.P., FORD-HUTCHINSON, A.W., SMITH, M.J.H. & WALKER, J.R. A sponge implantation test in the rat as a model for screening anti-inflammatory activity

472P BENDING, M.R., BENNETT, P.N. & ROWLAND, M. Pethidine pharmacokinetics in dog: dose and time studies

473P BARBHAIYA, R.H. & TURNER, P. Fluorimetric determination of ampicillin and cephalexin

474P BLAU, K., GOODWIN, B., WOODS, H.F. & YOUDIM, M.B.H. Phenylethylamine formation in perfused rat liver

475P CHICHMANIAN, R.M., KINSUN, I., MOULIN, M.A. & SAVINI, E.C. The effect of glucocorticoids on turn-over of acetylcholine and prostaglandins in the isolated guinea-pig ileum

*Br. J. Pharmac.* (1976), **58**, 323–331

# THE INHIBITORY EFFECT OF GALLAMINE ON MUSCARINIC RECEPTORS

A.L. CLARK & F. MITCHELSON
Department of Pharmacology, Victorian College of Pharmacy, Parkville, Victoria, Australia 3052

1 The inhibitory effect of gallamine (1.1 μM–1.1 mM) on negative inotropic responses to acetylcholine (ACh) or carbachol (CCh) was investigated in isolated electrically stimulated atria of the guinea-pig. Gallamine caused parallel rightward shifts of the dose-response curves to the agonists, with no depression of the maximal response.

2 Gallamine (0.11–1.1 mM) produced a greater degree of antagonism towards CCh than towards ACh. With either agonist, the degree of antagonism produced by gallamine in high concentrations was less than that expected for a competitive antagonist.

3 Similar findings were made when either negative inotropic or chronotropic responses were recorded in spontaneously beating guinea-pig atria. The inhibitory effect of gallamine against the negative inotropic response to cholinomimetics in electrically stimulated atria was not altered either in the presence of propranolol (17 μM) or in atria obtained from guinea-pigs pretreated with di*iso*propylphosphorofluoridate (DFP) (12.5 μmol/kg, in divided doses over 3 days).

4 When ACh was used as the agonist, combination of gallamine with atropine (0.05–0.4 μM) produced dose-ratios which were less than expected for combination of two competitive antagonists. The same phenomenon was observed in atria obtained from guinea-pigs pretreated with DFP.

5 It is suggested that the antagonism produced by gallamine is a type of non-competitive inhibition, which has been termed 'metaffinoid antagonism'. An antagonist of this type allosterically alters the affinity of the agonist for its binding site, rather than changing the effectiveness of the agonist-receptor interaction.

## Introduction

It has been reported that when gallamine is used clinically as a neuromuscular blocking agent, it produces tachycardia if used in conjunction with certain types of anaesthetics (Doughty & Wylie, 1951). Investigation of this effect has yielded various possible explanations. Brown & Crout (1966; 1968; 1970) showed that gallamine had a positive inotropic effect on mammalian cardiac muscle *in vitro* which was abolished by propranolol, or by pretreatment with reserpine. They suggested that gallamine possessed a sympathomimetic effect which was mediated by the release of noradrenaline from cardiac adrenergic nerve endings.

Gallamine also antagonizes negative inotropic and chronotropic effects produced by preganglionic vagal stimulation of the heart (Della Bella, Rognoni & Gopal, 1961; Brown & Crout, 1968). Della Bella *et al.* (1961) explained this effect as being due to a ganglion blocking action of gallamine in the heart. However, Riker & Wescoe (1951) showed that gallamine also inhibited acetylcholine (ACh) or methacholine-induced bradycardia. They postulated that the action of gallamine on the heart was like that of atropine, but it was a much less potent antagonist at other muscarinic sites.

More recent studies by Rathbun & Hamilton (1970) further emphasized the specificity of gallamine for the cholinoceptors of the heart. They supported the suggestion of an atropine-like action for gallamine, as the effects produced by gallamine fulfilled their requirements for competitive antagonism. Since atropine does not possess the selectivity for the heart exhibited by gallamine, it was decided to re-investigate the anti-muscarinic effects of gallamine and to elucidate further the nature of its antagonism of cholinomimetics.

## Methods

### *Guinea-pig isolated atria*

Spontaneously beating atria, or electrically stimulated left atria were mounted in a 10 ml organ bath filled with McEwen's solution (McEwen, 1956) maintained at 37°C and gassed with 95% $O_2$ and 5% $CO_2$. Left atria were stimulated by a square wave stimulator at supramaximal voltage, 2 ms pulse duration and a frequency of 3 Hz. Inotropic responses of the atria were recorded under a resting tension of 1 g via a Grass force-displacement transducer, FT 03C, on a Grass model 79 polygraph. In some experiments with spontaneously beating atria, chronotropic responses were monitored by a model 7P4D tachograph attachment on a polygraph.

### *Other tissues*

Guinea-pig isolated bladder and ileum were mounted in a 10 ml organ bath, under the same conditions as the atria. Contractions of the tissue were recorded by a force-displacement transducer on a polygraph.

### *Di-isopropylphosphorofluoridate (DFP) treatment*

Guinea-pigs were pretreated with subcutaneous injections of DFP for 3 consecutive days before use of the atria. Solutions of DFP in 0.9% w/v NaCl solution (saline) were prepared immediately before injection, each animal receiving 5 µmol/kg on the first and third day and 2.5 µmol/kg on the second day. On the fourth day, 24 h after the last injection, animals were killed and the atria removed for isolated tissue experiments or cholinesterase determinations.

### *Cholinesterase determinations*

Cholinesterase activity was determined by the pH-Stat method, using a Radiometer Automatic Titrator as described previously (Clark & Mitchelson, 1974). The substrate used was ACh (1 mM) and the titrant was NaOH (5 mM). In experiments to determine the anticholinesterase activity of gallamine, gallamine was incubated with the tissue for 20 min before the addition of substrate. Results are expressed as µmol NaOH $g^{-1}$ $min^{-1}$.

### *Experimental design*

In all experiments agonists were added for a period sufficient to ensure that the response to the administered concentration of drug had reached its maximum. For acetylcholine and adenosine triphosphate (ATP) a contact time of up to 1 min with the tissue was usually found sufficient together with a cycle time of 3 minutes. For CCh a contact time of 2 min was usually required with a cycle time of 5 minutes.

With these regimens, constant responses to agonists were obtained at least in duplicate. Responses were considered constant when 2 responses expressed as % inhibition differed by no more than 5%. Constant responses to at least 3 concentrations of agonist lying on the linear portion of the log dose-response line were obtained before addition of any antagonist. Following incubation with antagonists, responses to agonists were again obtained in duplicate, the duplicate response being recorded 15–30 min after the initial response. This procedure ensured that equilibrium conditions had been reached in the presence of the antagonist. The initial incubation period with the antagonist was 60 min for atropine and 20 min for gallamine or homatropine. The antagonist was replaced in the tissue bath fluid immediately after washing of the tissue. In experiments carried out in the presence of propranolol, the adrenoceptor antagonist was added to the bathing fluid reservoir at the beginning of the experiment.

In experiments involving combination of gallamine with atropine, responses in the presence of atropine alone were always determined first after establishment of a dose-response line to the agonist. Particular care was taken to ensure that the dose-ratio produced by atropine was determined under equilibrium conditions as described above. Gallamine (0.11 mM) was then added and following a further 20 min incubation, a third dose-response line was determined under equilibrium conditions in order to calculate the dose-ratio produced by the combination of the two antagonists.

In isolated bladder and ileum experiments, at least 3 concentrations of the agonist which produced between 20% and 80% of the maximum contractile response of the tissue were employed, both in the control period and after the addition of the antagonist.

### *Dose-ratio determinations*

Linear regression lines were fitted by the method of least squares to the linear portion of the log dose-response relationship both in the control period and when the antagonist was present. Dose-ratios were obtained by calculating the concentration of agonist producing 50% inhibition of the contraction or rate of the atria (or 50% of the maximum contractile response in bladder and ileum) in the absence and presence of antagonist.

### *Statistical comparisons*

Student's $t$ test (2-tailed) was used to compare groups of data, unless otherwise stated.

### *Drugs*

The drugs used were: acetylcholine chloride (Sigma), adenosine-5′-triphosphate (ATP) (Boehringer), atropine sulphate (Knoll, Sigma), carbachol (CCh) (Koch-Light), di*iso*propylphosphorofluoridate (DFP) (Sigma), gallamine triethiodide (May & Baker), homatropine hydrobromide (Sigma), mecamylamine hydrochloride (Merck, Sharp & Dohme), nicotine hydrogen tartrate (BDH), propranolol hydrochloride (ICI).

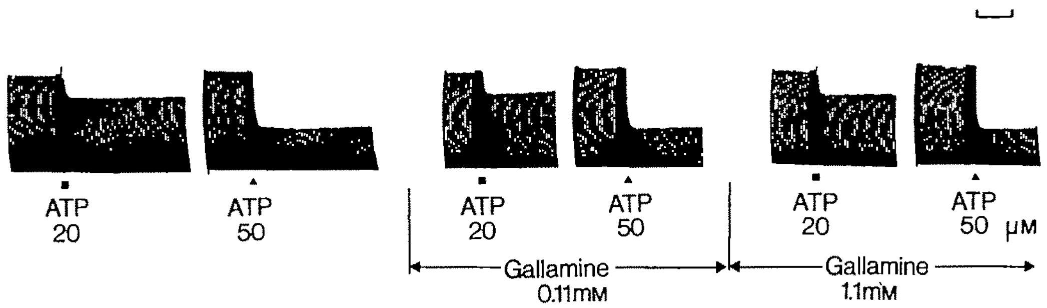


**Figure 1** The effect of gallamine 0.11 and 1.1 mM on the negative inotropic response to adenosine-5′-triphosphate (ATP) 20 µM (■) and 50 µM (▲) in an electrically stimulated left atrium of the guinea-pig. Time marker indicates 4 s at fast recording speed and 24 s at slow recording speed.

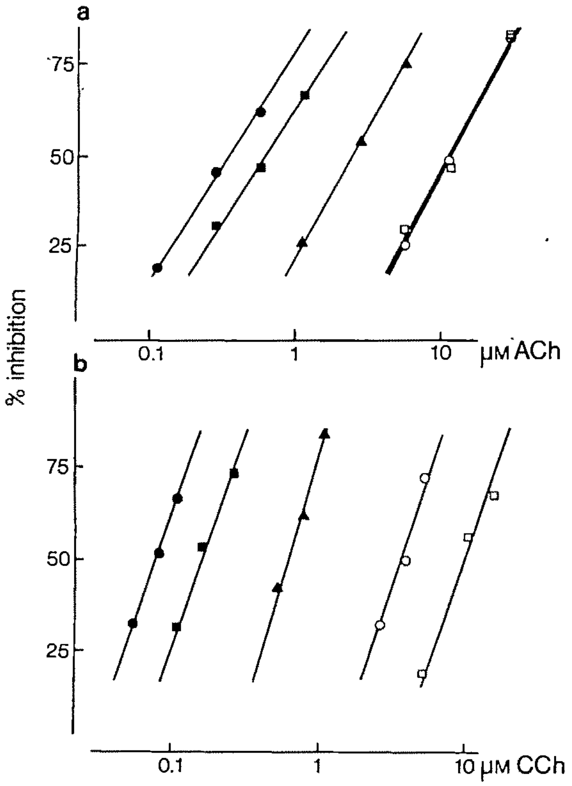


**Figure 2** The typical effect of gallamine on negative inotropic responses to (a) acetylcholine (ACh) and (b) carbachol (CCh) in electrically stimulated isolated atria of guinea-pig. Graphs are the results from a single experiment with each agonist and show the parallel displacement of the dose-response line to the agonist by increasing concentrations of gallamine (1.1 µM–1.1 mM). The points show the dose-response relationship obtained for each agonist in the absence of gallamine (●), and in the presence of gallamine 1.1 µM (■), 11 µM (▲), 0.11 mM (○) and 1.1 mM (□). The slopes of the regression lines obtained with each agonist in the presence of gallamine do not differ significantly ($P > 0.05$) from those obtained for each agonist in the absence of antagonist.

## Results

### *Isolated atrial preparations*

*Effect of gallamine.* Gallamine produced a transient increase in contractility which did not persist throughout the initial incubation period. With a concentration of 0.11 mM the initial increase in contractility was of the order of 10–20% but within 10–15 min the response had returned to pre-gallamine levels. The lack of any permanent effect on contractility may be seen in Figure 1. Similarly, in spontaneously beating preparations gallamine 0.11 mM produced a transient positive chronotropic response of the order of 10%.

*Effect of gallamine on responses to acetylcholine or carbachol.* In isolated driven left atria, gallamine (1.1 µM–1.1 mM) inhibited the negative inotropic responses to ACh or CCh. Gallamine produced parallel rightward shifts of the dose-response curve to either agonist, with no depression of the maximum response (Figure 2). However, gallamine at higher concentrations produced a lesser degree of antagonism than would be expected for a competitive antagonist, the degree of antagonism appearing to reach a limiting value at high concentrations which is more marked for ACh than for CCh. This can be readily seen when the results are plotted as a function of log [gallamine] against log (dose-ratio − 1) (Figure 3). When a linear regression line was fitted to the results for CCh, the slope of the line (0.78) was found to be significantly less ($P < 0.02$) than a theoretical value of 1.0 expected for competitive antagonism. In the case of ACh, where the slope was 0.46, the difference was highly significant ($P < 0.005$).

Similar results were obtained in spontaneously beating preparations when either inotropic or chronotropic responses were monitored. Using CCh as agonist there was no significant difference ($P > 0.05$) between the geometric mean dose-ratios obtained for negative inotropic responses in stimulated and spontaneously beating atria at any concentration of gallamine (1.1 µM–1.1 mM) investigated (Table 1).

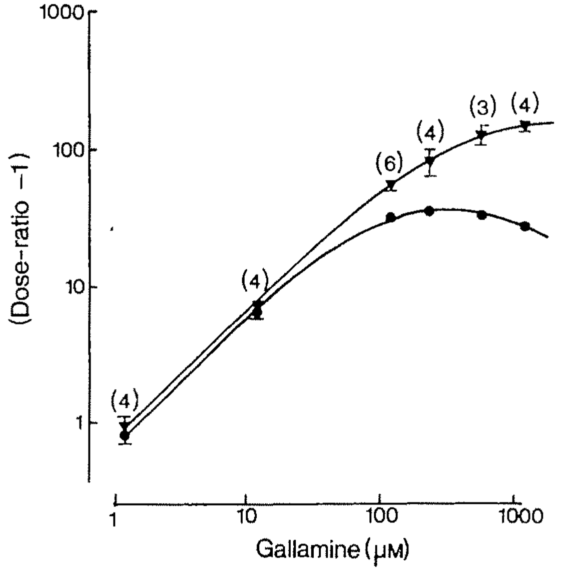


**Figure 3** Effect of gallamine on negative inotropic responses of electrically stimulated atria to acetylcholine (ACh) (●) or carbachol (CCh) (▼). Each point represents the mean of the log (dose-ratio−1) produced by a given concentration of gallamine, in four experiments with ACh as agonist. The number of experiments with CCh as agonist is shown in parentheses alongside each point. The vertical lines indicate s.e. mean, and where they are not shown they lie within the dimensions of the symbols.

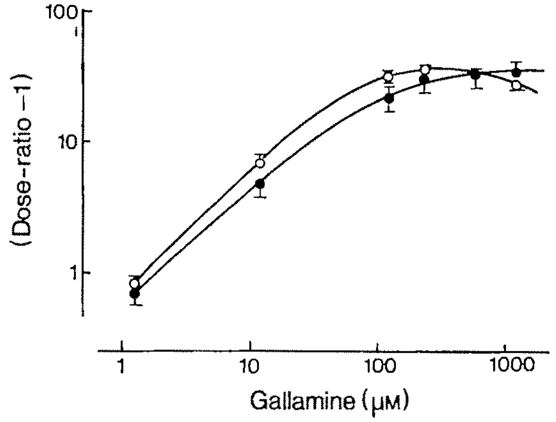


**Figure 4** Comparison of the inhibitory effect of gallamine on negative inotropic responses to acetylcholine (ACh) in electrically stimulated atria from control guinea-pigs (O) and in atria from animals pretreated with di-*iso*propylphosphorofluoridate (DFP, 12.5 μmol/kg over 3 days) (●). Each point for the latter curve is the mean of 5 experiments. The curve obtained with untreated animals is that shown in Figure 3. Other details as in Figure 3.

Similarly, dose-ratios obtained with gallamine against negative chronotropic responses produced by CCh did not differ significantly ($P > 0.05$) from those obtained for the negative inotropic response in electrically stimulated atria (Table 1). With ACh as agonist, gallamine also produced similar effects on negative inotropic responses to the agonist in both preparations (Table 1).

*Effect of gallamine on responses to nicotine.* Gallamine (1–11 μM) did not affect the positive inotropic response of electrically stimulated atria to nicotine (6–30 μM).

*Effect of gallamine on responses to ATP.* Gallamine in concentrations up to 1.1 mM had no effect on negative inotropic responses to ATP (0.01–1 mM) (Figure 1).

**Table 1** Comparison of the dose-ratios obtained with gallamine using either acetylcholine (ACh) or carbachol (CCh) in atrial preparations

| *Preparation* | *Response* | *Dose-ratio produced by gallamine 0.11 mM*† | | P†† |
|---|---|---|---|---|
| | | *CCh* | *ACh* | |
| Electrically stimulated | Inotropic | 60.8 (6)<br>(47.4–77.9) | 34.6 (4)<br>(26.9–44.4) | <0.05 |
| Spontaneously beating | Inotropic | 62.2 (6)<br>(51.5–74.9) | 40.1 (6)<br>(30.0–53.6) | <0.01 |
| | Chronotropic | 75.6 (6)<br>(37.3–153.4) | — | |

† Results are shown as geometric means (number of observations) together with the 95% confidence limits in parentheses below each mean. There is no significant difference ($P > 0.05$) between any dose-ratio obtained with any one agonist.

†† $P$ is the probability of the difference between values for CCh and ACh.

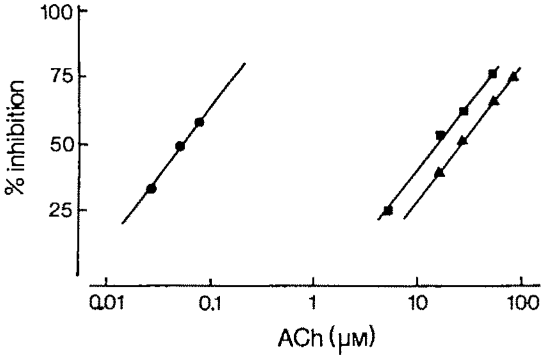


**Figure 5** Illustration of the results obtained from an experiment measuring the negative inotropic response to acetylcholine (ACh) in electrically stimulated atria obtained from guinea-pigs pretreated with di-*iso*propylphosphofluoridate (DFP). Each point is the mean response from at least 2 determinations in the absence of any antagonist (●), in the presence of atropine 0.4 μM (▲) and in the presence of atropine (0.4 μM) plus gallamine 0.11 mM (■). Note that the combination of atropine plus gallamine has resulted in a lesser degree of antagonism than that occurring in the presence of atropine alone.

*Effect of DFP.* In electrically stimulated atria obtained from animals pretreated with DFP, responses to ACh were potentiated 3.75 fold. The mean $ED_{50}$ (geometric) for ACh in untreated atria was 0.178 μM (95% confidence limits; 0.139–0.228, 19 experiments) and the corresponding value obtained in atria from DFP-treated animals was 0.047 μM (0.037–0.060, 28). However the dose-ratios produced by gallamine in these atria where acetylcholine was used as the agonist did not differ significantly ($P > 0.05$) at any concentration investigated from those obtained in atria from control animals (Figure 4).

*Effect of propranolol.* Similarly, the effect of gallamine on negative inotropic responses to CCh obtained in the presence of propranolol (17 μM) did not differ from controls.

*Effect of other antagonists.* The effect of homatropine (1 μM–0.3 mM) on negative inotropic responses to CCh in electrically stimulated atria was investigated. The relationship between log[homatropine] and log (dose-ratio − 1) was found to be a straight line of the type expected for a competitive antagonist, that is, with a slope (1.02) which did not differ significantly from 1.0 ($P > 0.5$). The geometric mean dose-ratios

**Table 2** Dose-ratios produced by atropine (0.01–0.4 μM) and gallamine (0.11 mM) alone and in combination, with acetylcholine as agonist*

| *Preparation*† *(n)* | *Atropine concentration (μM)* | *Atropine dose-ratio ($DR_{AC}$)* | *Gallamine (0.11 mM) dose-ratio ($DR_{AB}$)* | *Experimental combination dose-ratio ($DR_{ACB}$)* | *Expected combination dose-ratio ($DR_{AC}+DR_{AB}-1$)* | *P*†† |
|---|---|---|---|---|---|---|
| Spontaneous<br>(4) | 0.01 | 11.5<br>(10.7–12.4) | 40.1 (6)<br>(30.0–53.6) | 51.7<br>(43.2–61.8) | 50.6 | NS |
| Spontaneous<br>(3) | 0.1 | 100.9<br>(54.9–185.5) | | 93.9<br>(65.5–134.6) | 140.0 | <0.025 |
| Spontaneous<br>(6) | 0.2 | 208.4<br>(186.7–232.6) | | 163.4<br>(116.3–229.5) | 247.5 | <0.025 |
| Stimulated<br>(4) | 0.4 | 636.2<br>(491.8–823.0) | 34.6 (4)<br>(26.9–44.4) | 363.2<br>(206.2–638.3) | 669.8 | <0.025 |
| Stim./DFP<br>(3) | 0.05 | 28.4<br>(24.3–33.3) | 24.7 (5)<br>(12.9–47.0) | 36.4<br>(34.8–38.0) | 52.1 | <0.0005 |
| Stim./DFP<br>(3) | 0.2 | 247.5<br>(217.6–281.6) | | 193.7<br>(131.6–285.2) | 271.2 | <0.05 |
| Stim./DFP<br>(3) | 0.4 | 547.2<br>(173.2–1728.0) | | 351.2<br>(172.7–714.0) | 570.9 | <0.05 |

* Dose-ratio quoted are the geometric mean dose-ratios obtained together with the 95% confidence limits in parentheses below each mean value. The dose-ratio listed for gallamine alone is the geometric mean dose-ratio (plus no. of determinations and 95% confidence limits) obtained in separate experiments. The 'expected combination dose-ratio' is the expected dose-ratio which would be obtained if both antagonists acted competitively (Paton & Rang, 1965).

† Spontaneous = inotropic response in spontaneously beating whole atria, stimulated = electrically stimulated left atria, Stim./DFP = electrically stimulated left atria obtained from animals pretreated with DFP, (*n*) = number of experiments.

†† *P* denotes the level of significance associated with a *t* test (one-tailed) for whether $DR_{ACB} < (DR_{AC} + DR_{AB} - 1)$. NS = not significant.

obtained with homatropine 1, 10, 100 and 300 µM were 15.6 (95% confidence limits 11.6–21.0, 3 experiments); 121.2 (97.3–251.9, 3); 1357 (985.8–1867, 3) and 4910 (2117–11390, 3) respectively.

*Experiments with combinations of antagonists.* The effect of gallamine (0.11 mM) was investigated in the presence of various concentrations of atropine in either spontaneously beating or electrically driven atria.

With ACh as the agonist and using electrically stimulated atria from either normal or DFP-pretreated animals a combination of atropine plus gallamine always produced a lesser degree of antagonism than that expected for a combination of 2 competitive antagonists (Figure 5 and Table 2). This effect was also observed in spontaneously beating atria (Table 2). In all cases this effect was most marked when higher concentrations of atropine were employed. With a concentration of 0.4 µM atropine addition of gallamine always produced a leftward shift of the dose-response line obtained in the presence of atropine (Figure 5). The same phenomenon was frequently observed with 0.1–0.2 µM atropine.

With CCh as the agonist, addition of gallamine in the presence of atropine produced a further rightward shift of the dose-response line, the resulting shift being of a similar order or less than that expected for combination of 2 competitive antagonists (Table 3).

### *Cholinesterase determinations*

*Gallamine.* Gallamine 1 µM only slightly reduced the rate of hydrolysis of 1 mM ACh from 2.21 ± 0.24 (6) µmol NaOH $g^{-1}$ $min^{-1}$ (mean ± s.e. mean, number of observations) to 1.76 ± 0.15 (6) but gallamine 1 mM reduced the rate by 50% to 1.11 ± 0.13 (6).

*DFP.* The rate of hydrolysis of 1 mM ACh by atrial homogenates from untreated animals was found to be 2.15 ± 0.19 (4) µmol NaOH $g^{-1}$ $min^{-1}$ (mean ± s.e. mean, no. of observations). In atria obtained from DFP-treated animals the corresponding rate was 0.55 ± 0.08 (6) µmol NaOH $g^{-1}$ $min^{-1}$. Pretreatment of animals with DFP resulted therefore in a 74% reduction in the rate of hydrolysis of ACh.

### *Other tissues*

In guinea-pig isolated bladder and ileum, gallamine (1.1 µM–0.11 mM) had little inhibitory effect on contractile responses to ACh or CCh. With CCh as the agonist in ileum, gallamine (0.11 mM) produced a mean dose-ratio of 6.9 (95% confidence limits 2.5–19.3, 3 observations) and with ACh as the agonist the dose-ratio was 1.4 (0.6–3.2, 3). Similar findings were made in guinea-pig bladder. The value for CCh was 2.4 (2.1–2.8, 3) and for ACh was 1.4 (0.3–6.4, 3).

## Discussion

It has been repeatedly shown that gallamine antagonizes the effects produced by ACh, methacholine (MCh) or stimulation of the vagus nerve to the heart *in vitro* (Riker & Wescoe, 1951; Della Bella *et al.*, 1961; Brown & Crout, 1968; Rathbun & Hamilton, 1970). Our investigations have reconfirmed that in isolated atria of the guinea-pig, gallamine inhibits the negative inotropic and chronotropic responses produced by ACh or CCh. Della Bella *et al.* (1961) suggested that gallamine may produce its effects by a blockade of ganglionic transmission. However, this is unlikely to be the explanation of the

**Table 3** Dose-ratios produced by atropine (0.05–0.4 µM) and gallamine (0.11 mM) alone and in combination, with carbachol as the agonist in electrically stimulated atria†.

| *Number of experiments* | *Atropine concentration (µM)* | *Atropine dose-ratio ($DR_{AC}$)* | *Gallamine (0.11 mM) dose-ratio ($DR_{AB}$)* | *Experimental combination dose-ratio ($DR_{ACB}$)* | *Expected combination dose-ratio ($DR_{AC}+DR_{AB}-1$)* | P |
|---|---|---|---|---|---|---|
| 4 | 0.05 | 74.3<br>(54.2–101.7) | 60.8 (6)<br>(47.4–77.9) | 144.7<br>(83.8–260.2) | 134.1 | NS |
| 4 | 0.1 | 100.2<br>(97.5–103.0) | | 113.2<br>(93.3–137.6) | 160.0 | < 0.01 |
| 3 | 0.2 | 171.6<br>(114.1–258.2) | | 195.4<br>(125.4–304.3) | 231.4 | NS |
| 3 | 0.4 | 497.0<br>(411.4–600.5) | | 548.7<br>(216.0–139.4) | 556.8 | NS |

† Same details as for Table 2.

findings reported here as gallamine in concentrations up to 11 μM, did not antagonize responses to nicotine and in preliminary experiments mecamylamine (20 μM) had no effect on responses to CCh although it completely blocked positive and negative inotropic actions of nicotine. A non-specific antagonism of cardio-inhibitory agonists is excluded also because negative inotropic responses to ATP were not affected by gallamine in concentrations up to 1.1 mM.

Gallamine thus appears to have an antimuscarinic action in the atria, and exhibits some characteristics of a competitive antagonist as it produces parallel rightward shifts of the log dose-response curve to ACh or CCh, with no depression of the maximum response. However, when the results are plotted as a function of log[gallamine] against log(dose-ratio−1) the resultant plot differs from that expected with a competitive antagonist, that is a straight line with a slope of 1.0 (Arunlakshana & Schild, 1969). In the case of gallamine, the dose-ratios produced with the higher concentrations tend towards a limiting value and the upper part of the curve no longer follows a linear relationship. This effect was observed with either electrically stimulated or spontaneously beating atria and in the latter when either inotropic or chronotropic responses were monitored. Thus it is not due to any artifact brought about by electrical stimulation or to possible interference in the magnitude of inotropic responses by concomitant changes in spontaneous rate (Koch-Weser & Blinks, 1963).

Gallamine was found to be a much less potent antagonist than atropine, a concentration of 1 mM gallamine producing similar dose-ratios to those obtained with 0.1–0.2 μM of atropine when CCh was the agonist. It may be suggested that with the higher concentrations of gallamine employed (>0.1 mM) some other action of gallamine is reducing its effectiveness as a muscarinic antagonist and thus causes the decrease in the slope of the 'Arunlakshana-Schild' curves. For example, gallamine at high concentrations might depress atrial activity non-specifically and thereby facilitate drugs such as cholinomimetics which inhibit atrial contractility. However, gallamine did not directly depress contractility or rate and furthermore had no effect on the inhibitory response to ATP. Again, it may be suggested that high concentrations of all competitive antagonists may decrease the slope of the 'Arunlakshana-Schild' plot. However, homatropine, when used in comparable concentrations (1 μM–0.3 mM) to those employed with gallamine gave rise to larger dose-ratios as well as a linear 'Arunlakshana-Schild' plot.

Gallamine has been reported to possess anticholinesterase activity in high concentrations (Todrick, 1954), and in the atria, gallamine 1 mM reduced the rate of hydrolysis of ACh by 50%. As such anticholinesterase activity would counteract the antimuscarinic action of gallamine at high concentrations it is of interest that responses to CCh are not potentiated by pretreatment of the animals with DFP (Madden & Mitchelson, 1975) indicating that CCh does not mediate its action by releasing endogenous ACh. Also the anticholinesterase activity of gallamine cannot account for the smaller dose-ratios obtained when ACh was the agonist in place of CCh. Pretreatment with DFP, although inhibiting hydrolysis of ACh by 74%, did not markedly affect the maximum dose-ratio produced by gallamine against ACh. However, the anticholinesterase activity of gallamine may have been responsible for the observation that concentrations of 0.5–1 mM gallamine always produced smaller dose-ratios than a concentration of 0.2 mM as this effect was not observed after DFP pretreatment (Figure 4).

Another consideration is that CCh in high concentrations may release catecholamines (Barnett & Benforado, 1966; Löffelholz, 1970). However, such a release would reduce the effectiveness of CCh in producing negative inotropic responses at high concentrations and be manifested as an apparent increase in the dose-ratios produced by gallamine. Furthermore, gallamine itself may release catecholamines (Brown & Crout, 1968), which would also increase its effectiveness as an inhibitor of cholinomimetics. The presence of propranolol did not alter the dose-ratios indicating that sympathomimetic effects were not contributing to the inhibitory activity of gallamine on responses to cholinomimetics.

One explanation of findings reported here is that gallamine is acting allosterically to inhibit ACh or CCh. Antagonists which interact at a site which is distinct from but interdependent with the binding sites for agonists and cause a reduction in the affinity of agonists for their binding sites have been discussed by Ariëns, Van Rossum & Simonis (1956; 1964). Such an effect has recently been termed 'metaffinoid antagonism' (Offermeier & van den Brink, 1974). Like competitive antagonists and unlike noncompetitive antagonists of the 'mectactoid' type (Offermeier & van den Brink, 1974) a metaffinoid antagonist produces parallel shifts of the dose-response curve to the agonist without depression of the maximal response. However, metaffinoid antagonists differ from competitive antagonists in that their inhibitory activity at high concentrations reaches a limiting value when the antagonist has occupied all its available binding sites. The fact that gallamine at high concentrations produces greater inhibition of responses to CCh than to ACh would indicate that binding of these 2 agonists to the muscarinic receptor can be differentiated by metaffinoid antagonists.

The results from experiments involving combination of atropine and gallamine are also explainable in terms of gallamine being a metaffinoid antagonist. Such an antagonist will also affect the affinity of competitive antagonists for the muscarinic receptor and if the binding sites of competitive antagonists differ partly from those of agonists as has been suggested (Burgen, 1965) then metaffinoid antagonists may alter the affinities of agonists and competitive antagonist to differing extents. Thus using ACh as the agonist gallamine always reduced the degree of inhibition produced by atropine (Table 2 & Figure 5) suggesting that gallamine was reducing the affinity of atropine for its binding sites to a greater extent than it affected the affinity of ACh.

It should be noted that Stephenson & Ginsborg (1969) and Ginsborg & Stephenson (1974) have shown that combination of a fast-acting competitive antagonist with a slow-acting competitive antagonist may also lead to a reduction in the dose-ratio rather than an increase under certain conditions. Although gallamine acts more rapidly than atropine this does not appear to be the explanation in these experiments since Stephenson & Ginsborg (1969) found that the effect was only manifested when the agonist occupied a large proportion of the available receptors, that is, it functioned as a partial agonist. However, the experiments reported here with gallamine were conducted using ACh, a full agonist.

In the case of CCh, using gallamine and atropine in combination, the results (Table 3) indicate that gallamine reduces the affinity of both CCh and atropine to a similar extent. Although the combination dose-ratio is close to that predicted for two competitive antagonists, gallamine could also be acting as a metaffinoid antagonist which is equieffective against both CCh and atropine.

Previous workers such as Riker & Wescoe (1951) and Brown & Crout (1968) have concluded that gallamine has an atropine-like action on the heart because it inhibits negative inotropic and chronotropic responses to cholinomimetics in this tissue. Rathbun & Hamilton (1970), in a more extensive investigation, also concluded that gallamine was a competitive antagonist at muscarinic receptors for three reasons. Firstly, gallamine produced parallel rightward shifts of the dose-response curve to the cholinomimetic, with no depression of the maximum response. Secondly, Lineweaver-Burk plots of the effect of gallamine suggested that the antagonism was competitive. However, since Lineweaver-Burk plots involve the use of reciprocal values, they are not ideally suited for distinguishing between competitive and metaffinoid antagonists at high concentrations. Thirdly, Rathbun & Hamilton (1970) found that in pithed rats and spinal cats, combination of gallamine with atropine yielded a combined shift of the log dose-response curve to ACh which was closer to that expected for combination of two competitive antagonists, than for combination of atropine with a non-competitive (metactoid) antagonist. Thus, as there were only two possibilities considered, they concluded that gallamine was a competitive antagonist. However, examination of their data shows there was a less than expected effect from combination of gallamine and atropine with ACh as the agonist, which is in agreement with the findings in guinea-pig atria.

A number of bisquaternary compounds have been reported to possess antimuscarinic activity in a variety of tissues and many of these compounds have also been shown to possess properties different from those of competitive antagonists. These include toxogonin, TMB-4 (Kuhnen-Clausen, 1970; 1972; 1974), hemicholinium-3 (Bieger, Lüllmann & Wassermann, 1968; Mitchelson, 1971; Madden & Mitchelson, 1975) and a series of bisquaternary compounds related to the methonium series (Lüllmann, Ohnesorge, Schauwecker & Wassermann, 1965).

It has also been suggested that the interaction of gallamine with cholinesterases involves an allosteric effect (Changeaux, 1966; Roufogalis & Quist, 1972). Gallamine is a potent inhibitor of cholinesterase in solutions of low ionic strength and Changeaux (1966) found that inhibition of the hydrolysis of ACh by gallamine reached a limiting value, a finding which is analogous to our results on the muscarinic receptors in the atria. More recently, Roufogalis, Quist & Wickson (1973) have suggested that gallamine stabilizes one of two reversible configurations of acetylcholinesterase and that this can account for its effects on enzyme activity. A further point of interest concerning the effects of gallamine on acetylcholinesterase is that gallamine will antagonize the anticholinesterase activity of atropine when ACh is the substrate (Kato, 1971) an effect which bears a similarity to the results on atrial muscarinic receptors, with combination of gallamine and atropine using acetylcholine as the agonist.

Gifts of the following drugs are gratefully acknowledged: gallamine hydrochloride (May & Baker, Aust.), propranolol hydrochloride (ICI), mecamylamine hydrochloride (Merck, Sharp & Dohme).

**References**

ARIËNS, E.J., VAN ROSSUM, J.M. & SIMONIS, A.M. (1956). A theoretical basis of molecular pharmacology. *Arzneimittel Forsch*, **6**, 611–615.

ARIËNS, E.J., VAN ROSSUM, J.M. & SIMONIS, A.M. (1964). Drug receptor interaction: Interaction of one or more drugs with different receptor systems. In *Molecular Pharmacology*, Vol. 1, Medicinal Chemistry, Vol. 3, ed. Ariëns, E.J., Simonis, A.M. & Van Rossum, J.M. New York-London: Academic Press.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48–58.

BARNETT, A. & BENFORADO, J.M. (1966). The nicotinic effects of choline esters and of nicotine in guinea-pig atria. *J. Pharmac. exp. Ther.*, **152**, 29–36.

BIEGER, D.H., LÜLLMANN, H. & WASSERMANN, O. (1968). Über eine akute Anti-Acetylcholin-Wirkung von Hemicholinium No. 3 am Vorhof des Meerschweinchens. *Naunyn Schmiedebergs Arch. Pharmak. exp. Path.*, **259**, 386–393.

BROWN, B.R. & CROUT, J.R. (1966). Mechanism of the positive inotropic effect of gallamine on guinea-pig heart muscle. *Fedn Proc.*, **25**, 472.

BROWN, B.R. & CROUT, J.R. (1968). The sympathomimetic effect of gallamine on the heart. *Anaesthesiology*, **29**, 179–180.

BROWN, B.R. & CROUT, J.R. (1970). The sympathomimetic effect of gallamine on the heart. *J. Pharmac. exp. Ther.*, **172**, 266–273.

BURGEN, A.S.V. (1965). The role of ionic interaction at the muscarinic receptor. *Br. J. Pharmac. Chemother.* **25**, 4–17.

CHANGEAUX, J.-P. (1966). Responses of acetylcholinesterase from *Torpedo marmorata* to salts and curarizing drugs. *Mol. Pharmac.*, **2**, 369–392.

CLARK, A.L. & MITCHELSON, F. (1974). The effect of 4-(1-naphthylvinyl)-pyridine (NVP), a choline acetylase inhibitor, on autonomically innervated tissues of the rat. *Eur. J. Pharmac.*, **27**, 34–39.

DELLA BELLA, D., ROGNONI, F. & GOPAL, K. (1961). Curare-like drugs and cardiovagal synapses: comparative study *in vitro* on isolated guinea-pig vagus-heart preparation. *J. Pharm. Pharmac.*, **13**, 93–97.

DOUGHTY, A.C. & WYLIE, W.D. (1951). An assessment of flaxedil. *Proc. Roy. Soc. Med.*, **44**, 375–386.

GINSBORG, B.L. & STEPHENSON, R.P. (1974). On the simultaneous action of two competitive antagonists. *Br. J. Pharmac.*, **51**, 287–300.

KATO, G. (1972). Acetylcholinesterase. 1. A study by nuclear magnetic resonance of the binding of inhibitors to the enzyme. *Mol. Pharmac.*, **8**, 575–581.

KOCH-WESER, J. & BLINKS, J.R. (1963). Influence of the interval between beats on myocardial contractility. *Pharmac. Rev.*, **15**, 601–652.

KUHNEN-CLAUSEN, D. (1970). Investigations on the parasympatholytic effect of toxogonin on the guinea pig isolated ileum. *Eur. J. Pharmac.*, **9**, 85–92.

KUHNEN-CLAUSEN, D. (1972). Structure-activity relationship of mono- and bisquaternary pyridines in regard to their parasympatholytic effects. *Toxicol. Appl. Pharmac.*, **23**, 433–454.

KUHNEN-CLAUSEN, D. (1974). A novel approach to the questions of allosteric properties or a 'receptor reserve' of drug binding sites of intestinal smooth muscle cells. *FEBS Lett.*, **33**, 61–66.

LÖFFELHOLZ, K. (1970). Autoinhibition of nicotinic release of noradrenaline from post-ganglionic sympathetic nerves. *Naunyn Schmiedebergs Arch. Pharmak. exp. Path.*, **267**, 49–63.

LÜLLMANN, H., OHNESORGE, F.K., SCHAUWECKER, G.-C. & WASSERMANN, O. (1969). Inhibition of the actions of carbachol and DFP on guinea pig isolated atria by alkane-bis-ammonium compounds. *Eur. J. Pharmac.*, **6**, 241–247.

MADDEN, J. & MITCHELSON, F. (1975). The interaction of hemicholinium-3 (HC-3) with cholinomimetics and atropine. *Eur. J. Pharmac.*, **32**, 17–29.

McEWEN, L.M. (1956). The effect on the isolated rabbit heart of vagal stimulation and its modification by cocaine, hexamethonium and ouabain. *J. Physiol., Lond.*, **131**, 678–689.

MITCHELSON, F. (1971). Differentiation between the actions of acetylcholine and tetramethylammonium on the isolated taenia of the guinea-pig caecum by hemicholinium-3. *Br. J. Pharmac.*, **42**, 43–55.

OFFERMEIER, J. & VAN DEN BRINK, F.G. (1974). The antagonism between cholinomimetic agonists and $\beta$-adrenoreceptor stimulants. The differentiation between functional and metaffinoid antagonism. *Eur. J. Pharmac.*, **27**, 206–213.

PATON, W.D.M. & RANG, H.P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea pig in relation to acetylcholine receptors. *Proc. Roy. Soc. B.*, **163**, 1–44.

RATHBUN, F.J. & HAMILTON, J.T. (1970). Effect of gallamine on cholinergic receptors. *Can. Anaes. Soc. J.*, **17**, 574–589.

RIKER, W.F. & WESCOE, W.C. (1951). The pharmacology of flaxedil with observations on certain analogs. *Ann. N.Y. Acad. Sci.*, **54**, 373–392.

ROUFOGALIS, G.D. & QUIST, E.E. (1972). Relative binding sites of pharmacologically active ligands on bovine erythrocyte acetylcholinesterase. *Mol. Pharmac.*, **8**, 41–49.

ROUFOGALIS, G.D., QUIST, E.E. & WICKSON, V.M. (1973). Effect of ligands on a discontinuous temperature dependance of the decarbamylation reaction of erythrocyte acetylcholinesterase. *Biochim. biophys. Acta*, **321**, 536–545.

STEPHENSON, R.P. & GINSBORG, B.L. (1969). Potentiation by an antagonist. *Nature, Lond.*, **222**, 790–791.

TODRICK, A. (1954). The inhibition of cholinesterase by antagonists of acetylcholine and histamine. *Br. J. Pharmac. Chemother.*, **9**, 76–83.

*(Received July 14, 1975.*
*Revised April 6, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 333–339

# PROSTAGLANDIN ANTAGONISM BY SODIUM *p*-BENZYL-4-[1-OXO-2-(4-CHLOROBENZYL)-3-PHENYLPROPYL]PHENYL PHOSPHONATE (N-0164)

K.E. EAKINS

Departments of Ophthalmology and Pharmacology, College of Physicians and Surgeons,
Columbia University, New York, USA

V. RAJADHYAKSHA & R. SCHROER

Nelson Research and Development Company, Irvine, California, USA

1 The ability of sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl]phenyl phosphonate (N-0164) to antagonize contractions produced by prostaglandins $E_2$ and $F_{2\alpha}$ on isolated preparations of gerbil, rat and guinea-pig gastrointestinal muscle has been studied.

2 N-0164 was found to be a potent, partially selective prostaglandin antagonist in these isolated smooth muscle preparations. The blockade produced by N-0164 in the isolated stomach strip of the rat had some, but not all, the characteristics of a competitive antagonism.

3 N-0164 produced a dose-dependent decrease in tone in the rat stomach strip that was abolished by pretreatment of the preparation with indomethacin.

4 N-0164 prevented diarrhoea induced by prostaglandin $E_2$ in mice when given by intraperitoneal injection but was less effective when given orally.

5 N-0164 inhibited oedema induced with croton-oil and pyridine-ether in the mouse ear.

6 N-0164 delayed the onset of erythema following ultraviolet irradiation of guinea-pig skin only when an equimolar amount of pralidoxime chloride was added to the vehicle.

7 It is concluded that N-0164 is a potent, partially selective prostaglandin antagonist on several isolated smooth muscle preparations. N-0164 exhibits activity *in vivo* particularly following local application when problems associated with penetration and distribution are minimized.

## Introduction

Prostaglandin antagonists are compounds which selectively interfere with the actions of prostaglandins as opposed to compounds such as aspirin and indomethacin which are known to interfere with the formation of prostaglandins by inhibition of the enzyme complex known as prostaglandin synthetase (Ferreira, Moncada & Vane, 1971; Smith & Willis, 1971; Vane, 1971).

The prostaglandin antagonists available at the present time belong to three chemically unrelated classes, 7-oxaprostaglandins (Fried, Santhanakrishnan, Himizo, Lim, Ford, Rubin & Grigas, 1969), dibenzoxazepine hydrazides (Sanner, 1969) and certain polymers of phloretin phosphate (PPP) (Eakins, Karim & Miller, 1970). The prostaglandin antagonist activity of PPP was found to reside in the low molecular weight material (Eakins, 1971) and a number of compounds related to the monomers and dimers of PPP were synthesized and shown to possess prostaglandin antagonist activity (Eakins, Fex, Fredholm, Hogberg & Veige, 1972). A further series of phenyl phosphonates was synthesized of which sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl]phenyl phosphonate (N-0164, Figure 1) is an example. In the present study we describe the activity and selectivity of N-0164 as a prostaglandin antagonist on some smooth muscle systems *in vitro*. In addition, we have investigated the activity of this compound *in vivo* against prostaglandin $E_2$-induced diarrhoea by different routes of administration and the effectiveness of N-0164 as a topical anti-inflammatory agent.

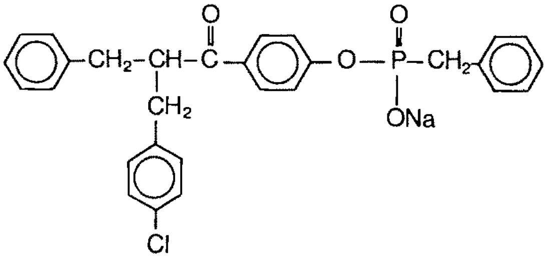


**Figure 1** Chemical structure of N-0164.

## Methods

### In vitro *smooth muscle preparations*

Muscle contractions were measured isotonically with a Brush isotonic muscle transducer coupled to a Heath Servorecorder (Model EU20B).

*Gerbil colon.* Gerbils (*Meriones shawi*) weighing between 50 and 150 g were stunned and bled. The middle part of the ascending colon was removed and suspended in a 5 ml organ bath containing de Jalon's rat colon solution (Ambache, Kavanagh & Whiting, 1965) at 28°C gassed with $O_2$. A dose-cycle of 3 to 4 min and a contact time of 2 min was used for all the agonists studied.

*Guinea-pig ileum.* The animals were stunned and bled. Segments of ileum were suspended in a 5 ml organ bath containing Tyrode solution at 37°C gassed with 5% $CO_2$ in $O_2$. A dose-cycle of 3 min and a contact time of 1 min was used for all agonists.

*Rat fundus.* Strips of rat fundus were prepared according to Vane (1957). The preparations were suspended in Krebs solution at 37°C bubbled with 5% $CO_2$ in $O_2$.

*Experimental procedure.* The activity and selectivity of the prostaglandin-blockade produced by N-0164 was determined by measuring the effect of the antagonist on contractions produced by a constant dose of various agonists. Doses of each agonist were chosen to yield a contraction between 40–60% of maximum. Constant responses were then established and N-0164 added to the bathing fluid and left in contact for 2 min before the addition of each agonist. The concentration of N-0164 was then increased stepwise until the contractions produced by the prostaglandins were antagonized approximately 100%. In a separate series of experiments on the rat fundus preparation, three point dose-response curves were established for each agonist in the absence and presence of the antagonist following a 15 min contact period. Horizontal shifts of the dose-response curves were measured at the level of the $ED_{50}$ and the dose-ratio calculated. The plot of log (dose-ratio − 1) against the negative log of the molar concentration of N-0164 was made according to Arunlakshana & Schild (1959) and the $pA_2$ value determined graphically.

### In vivo *studies*

*Inhibition of prostaglandin-induced diarrhoea.* Unfasted Swiss Webster mice, weighing between 16–18 g were used in groups of 10 animals. Prostaglandin $E_2$ (50 µg/kg i.p.) produced diarrhoea within 30 min in 95% of the animals. Other groups of animals were pretreated with various doses of N-0164 dissolved in 0.2 ml 0.9% w/v NaCl solution (saline) administered either orally or by intraperitoneal injection. Control animals were pretreated with an equal volume of saline. The dose of N-0164 required to protect 50% of the animals from diarrhoea ($ED_{50}$) was calculated.

*Croton oil-induced ear oedema.* Male Sprague-Dawley rats (50–100 g) were divided into groups of 6 animals. N-0164 was dissolved in saline and added to a mixture containing 4 parts pyridine and 5 parts ether containing 4% croton oil. The mixture was then added to one ear of each animal as described by Tonelli, Thibault & Ringler (1965). Control ears received the mixture without N-0164. Six hours later the animals were killed and a uniform tissue sample (No. 8 biopsy punch) taken from each ear and weighed. The difference in weight between biopsies taken from treated and control ears was then determined.

*Guinea-pig u.v. erythema.* Hartley guinea-pigs (male) were depilated 18 h before the start of the test. Animals were divided into groups of four and given a standard 30 s burn with a 360 W mercury vapour arc tube filtered with a vycor filter placed 11 cm from the animals' backs. Prior to burning, each animal was covered with a rubber sheet with two standard holes (1–2 cm in diameter) for exposure of the skin to the light. Following the burn, each animal was treated topically with an appropriate solution over the entire shaved surface. In the initial experiments the animals were either treated with a control vehicle (90% polyethylene glycol in $H_2O$) or different concentrations of N-0164 prepared in the polyethylene glycol vehicle.

Each animal was scored every hour for 6 h following treatment with each spot being scored according to the following scale: 0 = no observed redness; 1 = reddish tint without distinct outline; 2 = definite outline of reddish tint; 3 = bright red; 4 = small blister development.

## Results

The ability of N-0164 to reduce submaximal responses produced on the isolated gerbil colon and rat stomach strip by a variety of agonists was studied. The antagonist was added to the organ bath 2 min before each agonist and was present during the induced contraction. N-0164, 0.5 to 2 µg/ml (1 µM to 3.8 µM), antagonized responses to prostaglandins $E_2$ and $F_{2\alpha}$ of the gerbil isolated colon (Figure 2). At the higher concentrations of N-0164, contractions produced by angiotensin and bradykinin were depressed, although to a lesser extent than those produced by either prostaglandin. Contractions

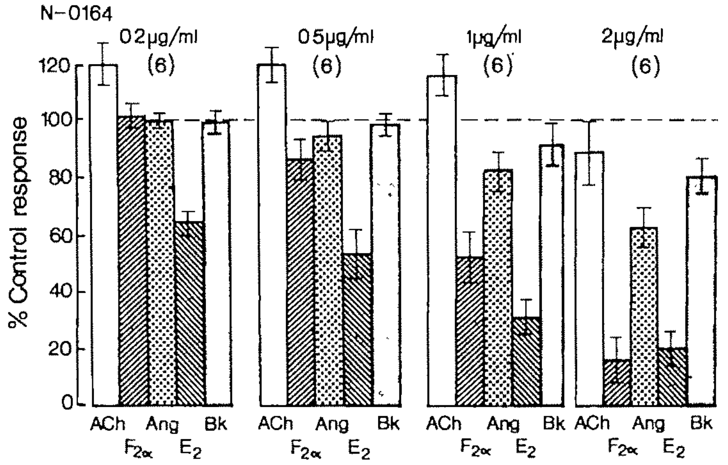


**Figure 2** Effect of N-0164 on contractions produced by acetylcholine (ACh), prostaglandins $E_2$ and $F_{2\alpha}$ ($E_2$, $F_{2\alpha}$), angiotensin (Ang) and bradykinin (Bk) on the gerbil isolated colon. Each column represents the mean value, vertical bars the s.e. mean. Numbers of experiments in parentheses.

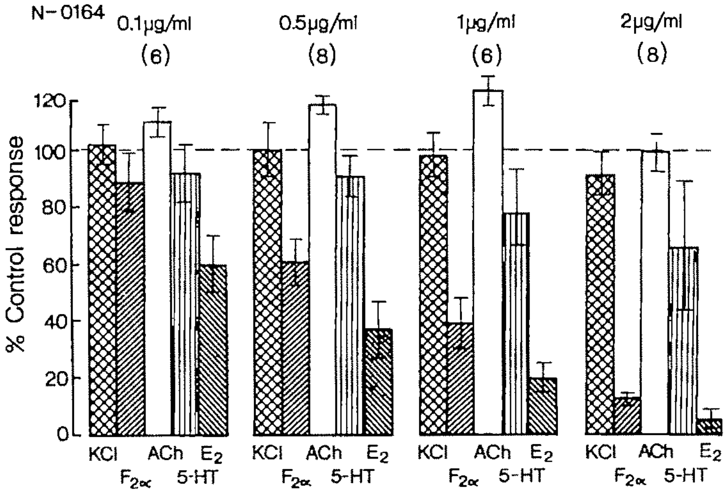


**Figure 3** Effect of N-0164 on contractions produced by potassium chloride (KCl), prostaglandins $E_2$ and $F_{2\alpha}$ ($E_2$, $F_{2\alpha}$), acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) on the stomach strip of the rat. Each column represents the mean value, vertical bars the s.e. mean. Numbers of experiments in parentheses.

produced by acetylcholine were not significantly reduced at any dose-level of N-0164. On the rat stomach strip preparation, contractions produced by prostaglandins $E_2$ and $F_{2\alpha}$ were markedly depressed by N-0164 at concentrations (0.19 µM to 3.8 µM) which did not consistently depress responses produced by acetylcholine. Some depression of responses produced by potassium chloride was noted at the higher dose-levels of N-0164 and these higher concentrations of N-0164 reduced responses produced by 5-hydroxytryptamine by some 30–40% (Figure 3). In all preparations the tone of the rat stomach strip preparation was reduced by N-0164 in a dose-dependent manner (Figure 4). In all experiments the responses to the agonists and in most experiments the baseline tone of each preparation returned to normal some 30 min after removal of N-0164 from the bathing fluid.

In six further experiments, preparations of the rat stomach strip were suspended in Krebs solution

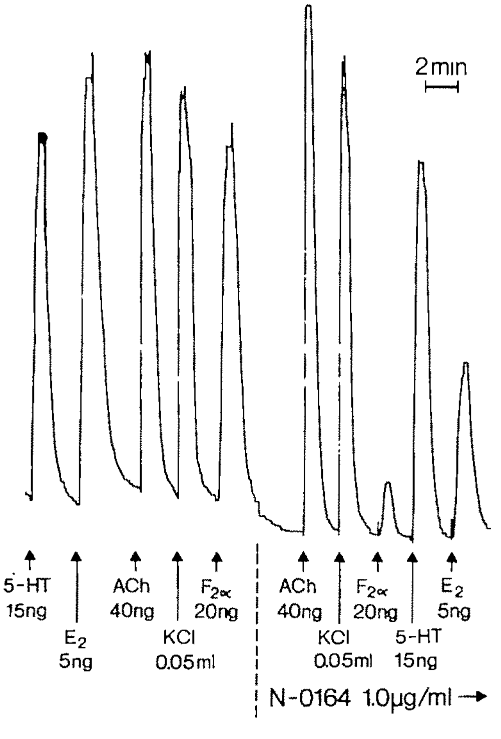


**Figure 4** Rat isolated stomach strip. Effect of N-0164 (1 µg/ml) on contractions produced by potassium chloride (KCl), prostaglandins $E_1$ and $F_{2\alpha}$ ($E_1$, $F_{2\alpha}$), acetylcholine (ACh) and 5-hydroxytryptamine (5-HT). Note the fall in baseline tension following the introduction of N-0164 into the bathing medium.

containing indomethacin (1 µg/ml) and in these preparations, N-0164 failed to reduce the tone at any dose-level (up to 5 µg/ml N-0164), although addition of isoprenaline (2 µg/ml) decreased the tone, indicating that the tissue was not fully relaxed in the presence of this concentration of indomethacin.

Experiments carried out on the guinea-pig isolated ileum showed that N-0164 was a potent antagonist of prostaglandin $E_2$ with an $IC_{50}$ value of 6.1 nM, as compared to values of 0.84 µM on the gerbil colon and 0.38 µM on the rat fundus preparation.

The antagonism by N-0164 of the responses of the rat stomach strip preparation to prostaglandins $E_2$ and $F_{2\alpha}$ appeared to be characteristic of a competitive antagonism, since in addition to the antagonism being reversible, parallel displacement of the dose-response curves to the right, without alteration of the maximum response produced by either prostaglandin $E_2$ or $F_{2\alpha}$, was observed over a wide range of antagonism. However, the shift of the dose-response curves to the right was not proportional to the increase in concentration of the antagonist. The dose-ratios for various agonists in this preparation at different concentrations of N-0164 are seen in Table 1. Dose-ratios of 138 and 153 were obtained for prostaglandins $E_2$ and $F_{2\alpha}$ respectively, in the presence of 9.5 µM N-0164, whereas the dose-ratio for 5-hydroxytryptamine was 10.95 and that for acetylcholine was essentially unaltered. The results from these experiments were plotted according to Arunlakshana & Schild (1959). This plot gave a $pA_2$ value of 6.7 for N-0164 against prostaglandin $E_2$ and 6.4 against prostaglandin $F_{2\alpha}$. The slope of the calculated regression line was −1.25 against prostaglandin $E_2$ and −1.45 against prostaglandin $F_{2\alpha}$.

Intraperitoneal injections of N-0164 dissolved in saline alone were found to prevent the diarrhoea produced in mice by the intraperitoneal injection of prostaglandin $E_2$. The $ED_{50}$ for N-0164 given by this route of administration was found to be 55 mg/kg. N-0164 was less active when given orally, with an $ED_{50}$ of 105 mg/kg.

N-0164 was found to inhibit chemically induced irritation (croton oil and pyridine-ether) in the mouse ear (Table 2). A minimum concentration of 1% N-0164 was required to produce a significant ($P < 0.025$) inhibition of oedema.

In other experiments, N-0164 was observed to delay the onset of erythema following ultraviolet

**Table 1** Antagonism of different agonists on the rat isolated stomach strip by N-0164

| Concentration† of N-0164 (µM) | Dose-ratio (A/a)* | | | |
|---|---|---|---|---|
| | Acetylcholine | $PGE_2$ | $PGF_{2\alpha}$ | 5-Hydroxytryptamine |
| 0.95 | 0.8 ± 0.1 | 5.0 ± 1.5 | 3.2 ± 0.2 | 2.0 ± 0.5 |
| 1.9 | 1.07 ± 0.07 | 17.5 ± 0.6 | 14.6 ± 2.6 | 2.1 ± 0.2 |
| 5.7 | 1.6 ± 0.2 | 50.75 ± 5.3 | 46.1 ± 4.5 | 11 ± 2.6 |
| 9.5 | 1.47 ± 0.02 | 138 ± 11 | 163 ± 8.4 | 10.95 ± 1.3 |

Results shown are the mean of four experiments ± s.e. mean. * A/a is the dose-ratio for each agonist calculated at the level of the $ED_{50}$. † The antagonist was allowed to remain in contact with the tissue for 15 min before determination of the dose-ratio using 3-point dose-response curves.

irradiation of guinea-pig skin. These experiments also demonstrated that the *in vivo* potency of N-0164 depends on the vehicle used since in the normal polyethylene glycol vehicle routinely used in this test, N-0164 was inactive at the three concentrations tested (1, 2 and 3%). However, when pralidoxime chloride (2-PAM) was added to the vehicle in equimolar amounts to N-0164, the ability of the antagonist to suppress erythema was observed (Table 3).

## Discussion

N-0164 has been found to be a potent, reversible, reasonably selective antagonist of contractile responses produced by both E and F prostaglandins on isolated preparations of the gerbil colon, rat stomach strip and guinea-pig ileum. The antagonism seen with N-0164 on the rat fundus had some of the characteristics of competitive blockade; the antagonism was reversible and the dose-reponse curves of both prostaglandins $E_2$ and $F_{2\alpha}$ were displaced to the right in parallel to a similar extent without any diminution of the maximum response over a 10-fold range of antagonist concentration. However, the shifts to the right of the dose-response curves were not proportional to the increase in concentration of the antagonist. Furthermore, the plots of log (dose-ratio – 1) against the negative log of the molar concentration of N-0164 yielded regression lines with slopes significantly greater than the theoretical value of 1.0 that would be expected from a simple bimolecular competitive antagonism. Further studies are required to resolve these discrepancies. However, the similarities between the dose-ratios obtained at the 50% maximal response for both prostaglandins at each concentration of N-0164 would suggest that prostaglandins $E_2$ and $F_{2\alpha}$ share a common site of action on the rat fundus preparation.

N-0164 was found to produce a dose-dependent decrease in tone in the rat stomach strip. This observation is similar to that obtained previously with another prostaglandin antagonist, SC 19220, on this

**Table 2** Topical anti-inflammatory activity of N-0164 in the croton oil mouse ear oedema test

| *Concentration of N-0164 (%)* | *% Increase in ear weight* |
|---|---|
| 0 | 93.5 ± 5.6 (17) |
| 0.3 | 87.5 ± 8 (15) |
| 1.0 | 72.4 ± 6.3 (16)* |
| 3.0 | 49.1 ± 4.6 (16)** |

Results expressed as mean with s.e. where *n* is number of experiments.
**P* < 0.025; ***P* < 0.005 (unpaired data, Students' *t* test).

**Table 3** Topical anti-inflammatory activity of N-0164 in ultraviolet erythema in guinea-pigs

| *Treatment after burn* | *Intensity of burn** | | | | | |
|---|---|---|---|---|---|---|
| | *1 h* | *2 h* | *3 h* | *4 h* | *5 h* | *6 h* |
| Vehicle† + 6% 2-PAM | 1 ± 0 (8) | 1.75 ± 0.16 (8) | 2.75 ± 0.16 (8) | 3.0 ± 0 (8) | 3.0 ± 0 (8) | 3.0 ± 0 (8) |
| Vehicle + 2% 2-PAM + 1% N-0164 | 1 ± 0 (8) | 1.75 ± 0.16 (8) | 2.75 ± 0.16 (8) | 2.9 ± 0.13 (8) | 3.0 ± 0 (8) | 3.0 ± 0 (8) |
| Vehicle + 5% 2-PAM + 2% N-0164 | 0.63 ± 0.18 (8) | 1.5 ± 0.19 (8) | 2.5 ± 0.19 (8) | 2.75 ± 0.16 (8) | 3.0 ± 0 (8) | 3.0 ± 0 (8) |
| Vehicle + 6% 2-PAM + 3% N-0164 | 0.25 ± 0.16 (8) | 0.63 ± 0.18 (8) | 1.4 ± 0.18 (8) | 2.25 ± 0.16 (8) | 2.5 ± 0.19 (8) | 2.88 ± 0.13 (8) |

* Intensity of the burn was scored as described in Methods section. † Control vehicle contained the highest concentration of pralidoxime chloride (2-PAM) used. N-0164 was without effect at the three concentrations tested in the absence of 2-PAM.

preparation (Bennett & Posner, 1971) and supports the concept (Bennett & Posner, 1971; Ferreira, Herman & Vane, 1972; Eckenfels & Vane, 1972) that prostaglandins contribute to the maintenance of tone in this and other isolated smooth muscle preparations. It is of interest that this action of N-0164 differs qualitatively from its antecedent, PPP, which produced dose-dependent contractions of the rat stomach strip (Bennett & Posner, 1971). The observation that pretreatment with indomethacin abolished the decreased tonus produced by N-0164 in the present experiments, together with the observation that 50 µM N-0164 did not inhibit prostaglandin synthetase derived from bovine seminal vesicles (R. Schroer, unpublished observations), is further evidence that this effect of N-0164 is mediated by blockade of the actions of endogenous prostaglandins.

Contractions produced by potassium chloride but not acetylcholine were somewhat reduced by the higher concentrations of N-0164 on the rat stomach strip. A reduction in contractions produced by potassium chloride by other prostaglandin antagonists has been described before (Bennett & Posner, 1971). The reason for this is not immediately apparent. The reduction of contractions produced by 5-hydroxytryptamine on the rat stomach strip and angiotensin and bradykinin on the gerbil colon by N-0164 in the present experiments may reflect a degree of involvement of prostaglandins in contractions produced by these substances (Chong & Downing, 1974; Frankhuijzen & Bonta, 1975; Crocker & Willavoys, 1976) but a definitive conclusion must await further investigation. However, it is interesting to note that although the dose-ratios for prostaglandin $E_2$ and $F_{2\alpha}$ increased with increasing concentrations of N-0164, the dose-ratio for 5-hydroxytryptamine increased a certain amount and then remained unaltered when the concentration of N-0164 was approximately doubled. Under the same experimental conditions the dose-ratio for acetylcholine remained unaffected. These experiments illustrate the importance of studying alterations in dose-ratios rather than changes in the effect of a single dose of an agonist when investigating a pharmacological antagonist.

N-0164 exhibited prostaglandin blocking activity *in vivo*. High doses were required to prevent diarrhoea induced by prostaglandin $E_2$ in mice, the compound being less effective orally ($ED_{50}$, 105 mg/kg) than when given by intraperitoneal injection ($ED_{50}$, 55 mg/kg). It should be noted that the $LD_{50}$ at 24 h in mice was 408 mg/kg intraperitonealy and 715 mg/kg orally (R. Schroer, unpublished observations).

N-0164 was more effective *in vivo* when problems associated with penetration and distribution were minimized, as in the croton oil-induced mouse ear oedema system. Further support for this proposal came from experiments on ultraviolet light-induced erythema in guinea-pig skin. N-0164 failed to influence either the intensity or duration of the erythema when applied topically in the normal polyethylene glycol vehicle. In contrast, when equimolar amounts of 2-PAM were added to the vehicle, N-0164 clearly suppressed the onset of the erythema. 2-PAM is thought to enhance penetration by a mechanism involving ion-pairing (F. Dea, personal communication). Prostaglandins have been implicated in the reaction of the skin to sunburn and ultraviolet irradiation (Greaves & Sondergaard, 1970; Mathur & Ghandi, 1972). Furthermore, a single topical application of indomethacin in a vehicle containing ethanol, propylene glycol and dimethylacetamide decreased the effects of sunburn in man for 24 h or more (Snyder & Eaglstein, 1974a). On the basis of their proposed mechanisms of action, N-0164 would be expected to have a shorter duration of action than indomethacin since the latter is an irreversible inhibitor of prostaglandin synthetase (Smith & Lands, 1971) and prostaglandins are thought to be synthesized continuously for some time after the exposure to ultraviolet irradiation (Snyder & Eaglstein, 1974b).

In conclusion, N-0164 was found to be a potent, partially selective prostaglandin antagonist *in vitro* in several isolated smooth muscle preparations. N-0164 exhibited activity *in vivo* particularly following local application when problems associated with penetration and distribution were minimized.

This work was supported in part by a grant from the U.S.P.H.S. (EY 00457). We thank Mrs Diane Reutter-Perez for excellent technical assistance and Dr John Pike of the Upjohn Company for generously supplying the prostaglandins used in this study.

## References

AMBACHE, N., KAVANAGH, L. & WHITING, J. (1965). Effect of mechanical stimulation on rabbits' eyes; release of active substances in anterior chamber perfusates. *J. Physiol., Lond.*, **176**, 378–408.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48–58.

BENNETT, A. & POSNER, J. (1971). Studies on prostaglandin antagonists. *Br. J. Pharmac.*, **42**, 584–594.

CHONG, E.K.S. & DOWNING, O.A. (1974). Reversal by prostaglandin $E_2$ of the inhibitory effect of indomethacin on contractions of guinea pig ileum induced by angiotensin. *J. Pharm. Pharmac.*, **26**, 729–730.

CROCKER, A.D. & WILLAVOYS, S.P. (1976). Possible involvement of prostaglandins in the contractile action of bradykinin on rat terminal ileum. *J. Pharm. Pharmac.*, **28**, 78–79.

EAKINS, K.E. (1971). Prostaglandin antagonism by polymeric phosphates of phloretin and related compounds. *Ann. N.Y. Acad. Sci.*, **180**, 386–395.

EAKINS, K.E., FEX, H., FREDHOLM, B., HOGBERG, B. & VEIGE, S. (1972). On the prostaglandin inhibitory action of polyphloretin phosphate. *Advances in the Biosciences*, **9**, ed Bergström, S. & Bernhard, S., pp. 135–138. Vieweg: Pergamon Press.

EAKINS, K.E., KARIM, S.M.M. & MILLER, J.D. (1970). Antagonism of some smooth muscle actions of prostaglandins by polyphloretin phosphate. *Br. J. Pharmac.*, **39**, 556–563.

ECKENFELS, A. & VANE, J.R. (1972). Prostaglandins, oxygen tension and smooth muscle tone. *Br. J. Pharmac.*, **45**, 451–462.

FERREIRA, S.H., HERMAN, A. & VANE, J.R. (1972). Prostaglandin generation maintains the smooth muscle tone of the rabbit isolated jejunum. *Br. J. Pharmac.*, **44**, 328–329P.

FERREIRA, S.H., MONCADA, S. & VANE, J.R. (1971). Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature, New Biol.*, **231**, 237–239.

FRANKHUIJZEN, A.L. & BONTA, I.L. (1975). Role of prostaglandins in tone and effector reactivity of the isolated rat stomach preparation. *Eur. J. Pharmac.*, **31**, 44–52.

FRIED, J., SANTHANAKRISHNAN, T.S., HIMIZO, J., LIM, C.H., FORD, S.H., RUBIN, B. & GRIGAS, E.O. (1969). Prostaglandin antagonists: synthesis and smooth muscle activity. *Nature, Lond.*, **223**, 208–210.

GREAVES, M.W. & SONDERGAARD, J. (1970). Pharmacologic agents released in ultraviolet inflammation studied by continuous skin perfusion. *J. Invest. Dermatol.*, **54**, 365–367.

MATHUR, G.P. & GHANDI, V.M. (1972). Prostaglandin in human and albino rat skin. *J. Invest. Dermatol.*, **58**, 291–295.

SANNER, J.H. (1969). Antagonism of Prostaglandin $E_2$ by 1-acetyl-2-(8-chloro-10,11-dihydrodibenz[b,f] [1,4] oxazepine-10-carbonyl hydrazine (SC 19220). *Arch. Int. Pharmacodyn. Ther.*, **180**, 46–56.

SMITH, J.B. & WILLIS, A.L. (1971). Aspirin selectively inhibits prostaglandin production in human platelets. *Nature, New Biol.*, **231**, 235–237.

SMITH, W.L. & LANDS, W.E.M. (1971). Stimulation and blockade of prostaglandin biosynthesis. *J. biol. Chem.*, **246**, 6700–6704.

SNYDER, D.S. & EAGLSTEIN, W.H. (1974a). Topical indomethacin and sunburn. *Br. J. Dermatol.*, **90**, 91–93.

SNYDER, D.S. & EAGLSTEIN, W.H. (1974b). Intradermal antiprostaglandin agents · and sunburn. *J. Invest. Dermatol.*, **62**, 47–50.

TONELLI, G., THIBAULT, L. & RINGLER, I. (1965). A bioassay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied corticoids. *Endocrinology*, **77**, 625–634.

VANE, J.R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Br. J. Pharmac. Chemother.*, **12**, 344–349.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature, New Biol.*, **231**, 232–235.

*(Received March 2, 1976.*
*Revised May 22, 1976)*

*Br. J. Pharmac.* (1976), **58**, 341–345

# GLUTAMATE ANTAGONISTS IN RAT HIPPOCAMPUS

M. SEGAL
Isotope Department, Weizmann Institute of Science, Rehovot, Israel

1 Hippocampal cellular responses to acidic amino acids and some of their antagonists were measured in the rat anaesthetized with urethane. The effects of these antagonists on the field responses of the rat hippocampus to afferent stimulation were measured in acute as well as chronically prepared rats.

2 Hippocampal pyramidal cells were excited by microiontophoretic application of glutamate and aspartate. These responses were antagonized by glutamic acid diethyl ester (GDEE), glutamic acid dimethyl ester (GDME) and by proline. Partial specificity could be seen as excitatory responses to acetylcholine were less susceptible to the antagonists.

3 Field responses of the hippocampus to commissural stimulation were reduced significantly in both an acutely prepared or the conscious rat following parenteral administration of GDEE and GDME but not proline. Responses to perforant path stimulation were not affected by these drugs.

4 It is suggested that an acidic amino acid may serve as a neurotransmitter in the commissural path to area CA1 of the dorsal hippocampus.

## Introduction

The acidic amino acids, glutamate and aspartate, have long been suggested as putative neurotransmitters in the nervous system (Curtis, Duggan, Felix, Johnston, Tebēcis & Watkins, 1972). They exert potent excitatory action towards neuronal action potentials when applied iontophoretically (Curtis *et al.*, 1972; Johnson, 1972) in nearly every part of the brain tested. Several antagonists to the action of glutamate have been suggested; glutamic acid diethyl ester (GDEE) among them has been reported to reduce the excitatory effects of glutamate without affecting responses to acetylcholine (ACh) in spinal cord, cuneate nucleus, ventrobasal thalamus, lateral geniculate and cerebral cortex (Curtis *et al.*, 1972; Haldeman & McLennan, 1972). Furthermore, GDEE blocks excitatory action of input pathways to some of these structures when applied iontophoretically (Haldeman & McLennan, 1972) or parenterally (Stone, 1973). Glutamic acid dimethyl ester (GDME) is a glutamate uptake blocker (Haldeman & McLennan, 1973) and has been shown in various preparations to potentiate cellular responses to glutamate. Its iontophoretic application in the hippocampus might be expected to produce effects opposite to those produced by GDEE. Recently, Van Harreveld & Fifkova (1973) have suggested that proline may antagonize glutamate responses in chicken isolated retina and elsewhere, whereas Felix & Künzle (1974) claimed that proline is a putative inhibitory transmitter in the cerebellum. A test of the efficacy of this compound in antagonizing acidic amino acid excitation was also attempted.

The hippocampus receives three main extrinsic excitatory inputs, two of which utilize neurotransmitters of unknown species. In this study the responses of hippocampal cells to acidic amino acids were tested. An attempt was made to antagonize these responses with GDEE, GDME and proline and the suceptibility of afferent responses to these antagonists was tested.

## Methods

Adult (200–300 g) male Wistar rats of a local breeding colony were used. Two types of experiments were performed using the acutely anaesthetized and the freely moving conscious rat.

Rats prepared for the acute experiments were anaesthetized with urethane (1 g/kg) and placed in a stereotaxic frame. The skin overlying the skull was reflected and a hole 2 mm in diameter drilled 3.5 mm posterior to bregma and 1.5 mm lateral to the midline suture. The dura was removed and the cortex covered with warm 3% agar. A 5-barrel micropipette 4–6 μm in diameter was inserted to a depth corresponding to that of the pyramidal cell layer of area CAI of the dorsal hippocampus. A bipolar concentric electrode was inserted at a similar point in the contralateral hippocampus. Another electrode was placed in the entorhinal cortex, the source of the perforant path to the hippocampus. The localization of the electrodes was verified histologically.

Three of the four outer pipettes of the 5-barrel micropipette were filled with the following compounds: acetylcholine chloride (2.5 M, Calbiochem), L-aspartic acid (Merck, 0.1 M, pH 8.0), DL-glutamic acid (K & K Laboratories, pH 8, 0.1 M), L-glutamic acid diethyl ester hydrochloride (GDEE, 0.1 M, Sigma), L-glutamic acid dimethyl ester hydrochloride (GDME, 0.1 M, Sigma) and L-proline (0.1 M, Fluka AG). The fourth barrel was filled with 5 M NaCl and served for testing current effects and to neutralize tip currents (Geller & Woodward, 1972).

The neuronal signals were recorded with the centre barrel which was filled with 5 M NaCl. The signals were fed via a preamplifier into an amplifier-filter which, in one channel, amplified the high frequency band (1–10 kHz) and recorded spike potentials. This amplifier was connected to a spike-height window discriminator and a ratemeter which displayed on a chart recorder integrated unitary firing rates.

In the other channel a low frequency band (1–1 kHz) for measuring evoked field potentials was amplified and connected to an Ortec signal averager which was set to average 16–32 traces. The hippocampal afferents were stimulated with 0.2 ms 40–100 μA monophasic pulses delivered through a battery operated simulator which was triggered by a Devices Digitimer which also triggered the signal averager.

The rats prepared for the chronic experiments were anaesthetized with sodium pentobarbitone (Nembutal, 50 mg/kg) and implanted with twisted 100 μm bipolar electrodes aimed at the entorhinal cortex and the hippocampus contralateral to the recording site where 62 μm microwire was implanted for recording. All wires were cemented to the skull and assembled in a plastic cap. After 3–4 days for recovery the rat was introduced into the test chamber, connected to a ten wire cable which delivered the stimuli to the rat and the responses through a preamplifier to the recording system described above. After termination of the experiments the rats were injected with an overdose of Nembutal perfused with 10% buffered formaldehyde and, at a later stage, sectioned on a freezing stage for determination of electrode placements. Further details of the methods are given elsewhere (Segal, 1973).

## Results

As already reported (Biscoe & Straughan, 1966), glutamate caused an increase in firing rates of hippocampal cells when ejected with very low currents (0–10 nA). All 57 cells tested were excited with a short latency (less than 2 s) compared to the latency of the excitatory action of acetylcholine (ACh, 3–5 seconds).

The effects of glutamate were also of shorter duration than those of ACh and often an apparent inhibition of spontaneous firing rates which follows an initial excitatory response to glutamate was observed. This inhibition was often accompanied by a reduction of spike size. Responses similar to those produced by glutamate in both the current needed to generate an excitation and the time course of the effects resulted from iontophoretically applied aspartate.

The effects of GDEE on the excitatory action of glutamate were tested in 27 cells. On most cells tested (Table 1) GDEE exhibited a potent direct inhibitory action accompanied by a reduction of spike size when applied with a medium to high current (40–80 nA). However, when applied with low to moderate currents (20–40 nA) it had no noticeable effect on spontaneous activity or spike configuration and with such a dose it antagonized the responses to glutamate in 20 of 27 cells tested (Figure 1). GDEE action was considered antagonistic when it caused at least a 50% reduction in response to glutamate in three successive test sequences. Its effects were relatively specific in the current range used; although some antagonistic action towards the excitatory responses of 6 cells to ACh was noticed, a complete blockade of cellular responses to ACh was seen in only one cell.

GDME had a direct excitatory action towards most of the cells tested without affecting spike size (Table 1), as might be expected from its presumed glutamate uptake blocking action. When tested against the effects of glutamate, it did antagonize the action of glutamate in 7 of 13 cells tested. This

**Table 1** Effects of glutamic acid diethylester (GDEE), glutamic acid dimethyl ester (GDME) and proline on the responses of hippocampal cells to glutamate (Glu) and acetylcholine (ACh) and on their spontaneous activity

| | *Glu* | | *ACh* | | *Direct action* | | |
|---|---|---|---|---|---|---|---|
| | *Block* | *No effect* | *Block* | *No effect* | *Increase* | *No effect* | *Decrease* |
| GDEE | 20 | 7 | 1 | 5 | 0 | 12 | 15 |
| GDME | 7 | 6 | 2 | 2 | 6 | 6 | 1 |
| Proline | 14 | 2 | 3 | 2 | 1 | 11 | 4 |

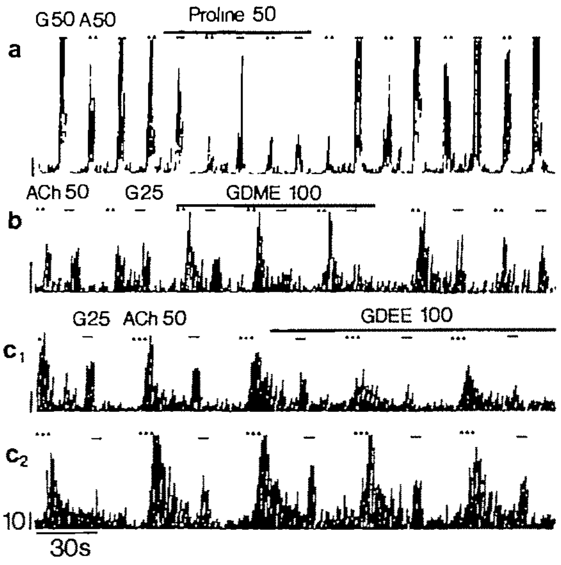


**Figure 1** Effects of (a) proline, (b) glutamic acid dimethyl ester (GDME) and ($c_I c_{II}$) glutamic acid diethyl ester (GDEE) on excitatory cellular responses to putative neutrotransmitters. (a) Cumulative spike records comparing the effects of glutamate (G) and aspartate (A) on cellular firing rates and the effects of proline on these responses. Drugs were applied at regular intervals for a duration indicated by a bar with an ejection current in nA indicated by the numbers above the bars (50 nA for glutamate). Note the slightly longer recovery time needed for aspartate responses compared with responses to glutamate. (b) Comparison of the effects of glutamate with those of acetylcholine (ACh) and the action of GDME towards these excitatory actions. Note the apparent increase of ACh responses during GDME administration along with the complete blockade of glutamate responses and the relative absence of effects on spontaneous firing rates of GDME. ($c_I c_{II}$) Two successive records of the effects of GDEE towards glutamate and ACh. An apparent antagonistic action of GDEE towards both ACh and glutamate can be seen. No direct action on the spontaneous firing rates was seen. Note the slower recovery of responses to glutamate as compared with those of ACh. In all traces the abscissa scale denotes time (30 s bar) and the ordinate scale, spikes per s (10 spikes per s bar).

proportion is far lower than that produced by GDEE towards glutamate excitation. In 2 of 4 cells tested the action of ACh was at least partially antagonized by GDME.

Proline seemed to have the least direct action on spontaneous cell activity and spike configuration among the drugs tested. Most of the cells (11 of 16 cells) were not directly affected by proline. Proline exerted a potent antagonistic action towards the effect of glutamate; in 14 of 16 cells tested the responses to glutamate were at least partially antagonized by proline. The effects of proline were also tested against aspartate in three cells. Proline antagonized responses to aspartate in all of these cells. When tested simultaneously, proline appeared to have a slightly better antagonistic action towards aspartate than towards glutamate (Figure 1a).

Hippocampal CA1 responses to commissural stimulation consisted of a large negative potential with a latency of 16–24 ms, recorded in stratum radiation, below the pyramidal cell layer. This potential is generated in the terminal area of the commissural path and its magnitude and polarity varies within the hippocampus (Andersen, 1960). When applied iontophoretically neither of the three glutamate antagonists could block the responses to stimulation of the commissural pathway without a primary potent direct action towards the spontaneous activity of the recorded cells. Therefore no further attempts to antagonize the responses to stimulation of the input pathways by iontophoretic administration of glutamate antagonists were made and instead the antagonists were administered parenterally. Intraperitoneal administration of GDEE, GDME and proline was attempted in four of the above rats. In three of them the evoked field responses to commissural stimulation were reduced to 30–50% of control (Figure 2) with little effect on the responses to perforant path stimulation. GDME had a similar potency in these three rats in that it reduced responses to commissural stimulation to 40–70% of control values without affecting perforant path responses. Proline has no effect at the dose level used (200 mg/kg i.p.).

The conscious rat preparation has the advantages of allowing the effects of drugs to be tested without interference of anaesthetics and enabling comparative tests of various drugs to be made since the original response to afferent stimulation can be maintained over several days (Segal, unpublished observations). The effects of GDEE, GDME and proline were tested in 6 rats. Both GDEE and GDME reduced selectively the commissural responses within 2–5 min after injection (Figure 3), while proline, as in the acute experiments, was without effects. The action of GDEE was pronounced (60–80% decrease in the commissural response) in one rat, moderate (20–25% decrease in the response magnitude) in 4 more rats and ineffective in one rat. GDME action was pronounced in two rats, moderate in one rat and ineffective in the other two rats tested. There were no conspicuous effects of these drugs on the behaviour of the tested rats.

## Discussion

The present results indicate that hippocampal neurones are excited by glutamic acid and that GDEE

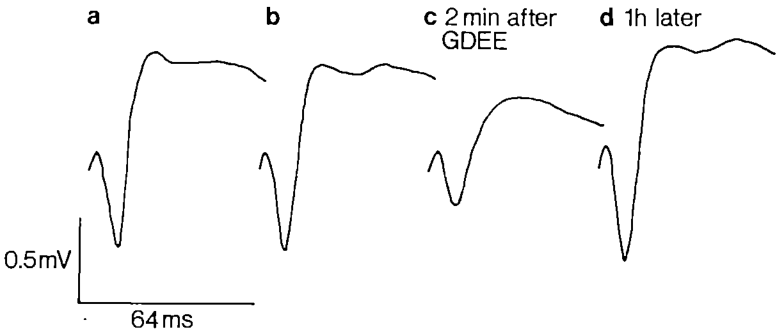


**Figure 2** Mean evoked commissural responses in an anaesthetized rat. Each trace is the mean response to 16 stimuli (4 V, 0.2 ms duration, 1 Hz frequency). The recording electrode was placed just below the pyramidal cell layer. A stable response (a & b) was reduced to (c) less than 50% of the control value 2 min after intraperitoneal injection of 200 mg/kg glutamic acid diethyl ester (GDEE). (d) Complete recovery was seen 1 h later. In this figure positivity is upwards.

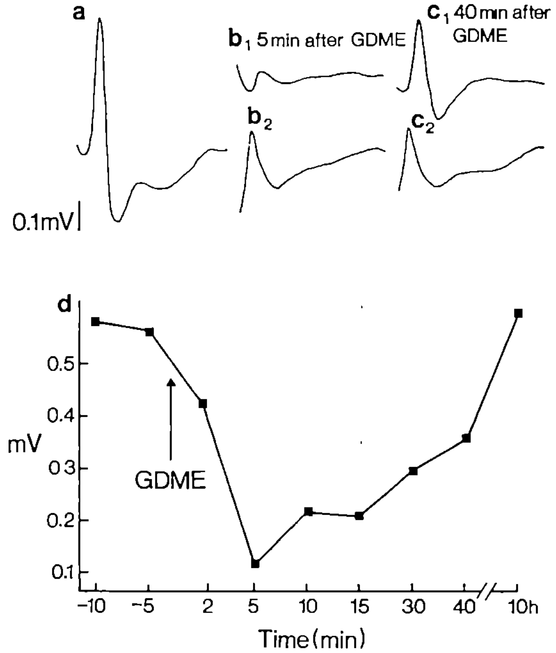


**Figure 3** Effects of glutamic acid dimethyl ester (GDME) on commissural responses in the conscious rat. (a) Mean response to commissural stimulation before drug administration; (b) 5 min after GDME injection: (b$_1$) response to commissural stimulation; (b$_2$) response to entorhinal (perforant path) stimulation; (c$_1$ and c$_2$) responses to commissural and entorhinal stimulation respectively, 40 min after drug injection. In all these records negativity is upwards. (d) Time course of the changes in the magnitude of the commissural response after GDME injection.

antagonizes these excitatory responses. Another drug known to interact with glutamate, GDME, previously reported to block glutamate uptake and potentiate glutamate responses (Haldeman & McLennan, 1973) also had an antagonistic action towards glutamate in the present experiments. Similarly, proline, an amino acid reported to have some glutamate antagonistic action (Van Harreveld & Fifkova, 1973) exhibited this effect in the present study.

When the effects of these drugs on responses to stimulation of pathways afferent to the hippocampus were tested, it was found that two of them, GDEE and GDME, had a selective antagonistic action, in at least some cases, towards the responses to the commissural input. This effect could not be demonstrated when the antagonists were administered iontophoretically. The separation between the recording site, at or near the pyramidal cell layer of field CA1 of dorsal hippocampus, and the terminal field of the commissural path is some 100–500 µm. It is therefore unlikely that sufficient amounts of the antagonist could have arrived at the terminal field and have a selective antagonistic action towards glutamate terminals. Parenteral drug application, on the other hand, presents other problems of interpretation, since the drug may also change the excitability of the presynaptic fibres or the stimulated area or even the recorded cells in a way that may not affect the responses to the other input pathway tested, the perforant path. Tests for these possibilities are certainly pertinent.

It is interesting to note, in this context, that although proline was a potent antagonist of glutamate responses, it had little effect on the response to the stimulation of the pathway. It is possible that proline, being a natural amino acid, was taken up mainly by other mechanisms and only a little of it arrived at the site where antagonistic action towards the pathway could be manifested.

The evidence for a transmitter role for glutamate or aspartate in the hippocampus is as yet incomplete. It has been reported thus far that aspartate uptake is reduced in decommisurized brain slices indicating that aspartate may be involved in commissural transmission (Nadler, Vaca, Cotman & Lynch, 1975). It was also suggested that glutamate is released upon stimulation of an intrinsic hippocampal pathway (Crawford & Connor, 1973). Recently, Spencer, Gribkoff, Cotman & Lynch (1976) have also reported that GDEE antagonizes hippocampal CAl cellular responses to glutamate and aspartate. They, too, were unable to differentiate between glutamate and aspartate.

Finally, Iversen & Storm-Mathisen (1976) found a selective uptake of [$^3$H]-glutamate into excitatory terminals which belong, in part, to the commissural path. Obviously with the small selection of putative excitatory neurotransmitters available for study it is almost inevitable that at least one of the two extrinsic excitatory and two intrinsic excitatory synapses in the hippocampus would utilize an acidic amino acid as a neurotransmitter. Our data in agreement with Nadler *et al.* (1975) supports the suggestion that glutamate or aspartate might be the transmitter of the commissural path. With the present lack of specific antagonists to glutamate or aspartate a more definite suggestion cannot be made on the basis of the present data. Further investigation is needed to obtain evidence which will allow a comparison of responses to both putative neurotransmitter application and stimulation of the pathway at the membrane level.

This research was supported by a grant from the United States–Israel Binational Foundation (BSF), Jerusalem, Israel. I would like to thank A. Edelson and V. Greenberger for his help and cooperation.

## References

ANDERSEN, P. (1960). Interhippocampal impulses. II. Apical dendritic activation of CAl neurons. *Acta physiol. scand.*, **48**, 178–208.

BISCOE, T.J. & STRAUGHAN, D.W. (1966). Microelectrophoretic studies of neurons in the cat hippocampus. *J. Physiol., Lond.*, **183**, 341–359.

CRAWFORD, I.L. & CONNOR, J.D. (1973). Glutamate in the cat hippocampus: Distribution and release. *Fedn Proc.*, **32**, 696.

CURTIS, D.R., DUGGAN, A.W., FELIX, D., JOHNSTON, G.A.R., TEBĒCIS, A.K. & WATKINS, J.C. (1972). Excitation of mammalian central neurons by acidic amino acids. *Brain Res.*, **41**, 283–301.

FELIX, D. & KÜNZLE, H. (1974). Iontophoretic and autoradiographic studies on the role of proline in nervous transmission. *Pflügers Archiv*, **350**, 135–144.

GELLER, H.M. & WOODWARD, D.J. (1972). An improved constant current source for microiontophoretic drug application studies. *EEG J.*, **33**, 430–432.

HALDEMAN, S. & McLENNAN, H. (1972). The antagonistic action of glutamic acid diethylester towards amino acid induced and synaptic excitations of central neurons. *Brain Res.*, **45**, 393–400.

HALDEMAN, S. & McLENNAN, H. (1973). The action of two inhibitors of glutamic acid uptake upon amino acid induced and synaptic excitations of thalamic neurons. *Brain Res.*, **63**, 123–129.

IVERSEN, L.L. & STORM-MATHISEN, J. (1976). Uptake of $^3$H-glutamate in excitatory nerve endings in the hippocampal formation of the rat. *Acta physiol. scand.*, **96**, 22A.

JOHNSON, J.C. (1972). Glutamic acid as a synaptic transmitter in the nervous system. A review. *Brain Res.*, **37**, 1–19.

NADLER, J., VACA, K., COTMAN, C.W. & LYNCH, G.S. (1975). Calcium dependent efflux of aspartate, glutamate and GABA from rat hippocampal formation: effects of selective lesions. *5th Neuroscience Meeting*, Abstracts, New York, 400.

SEGAL, M. (1973). Flow of conditioned responses in the limbic telencephalic system of the rat. *J. Neurophysiol.*, **36**, 840–854.

SPENCER, H.J., GRIBKOFF, V.K., COTMAN, C.W. & LYNCH, G.S. (1976). GDEE antagonism of iontophoretic amino acid excitations in the intact hippocampus and in the hippocampal slice preparation. *Brain Res.*, **105**, 471–481.

STONE, T.W. (1973). Cortical pyramidal tract interneurons and their sensitivity to l-Glutamic acid. *J. Physiol., Lond.*, **233**, 211–225.

VAN HARREVELD, A. & FIFKOVA, E. (1973). Effects of amino acids on the isolated chicken retina and on its response to glutamate stimulation. *J. Neurochem.*, **20**, 947–962.

*(Received March 19, 1976.*
*Revised May 12, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 347–357

# ANAESTHETICS DEPRESS THE SENSITIVITY OF CORTICAL NEURONES TO L-GLUTAMATE

C.D. RICHARDS & J.C. SMAJE
National Institute for Medical Research, Mill Hill, London NW7 1AA

1 The effects of general anaesthetics on the responses of neurones to iontophoretically applied L-glutamate have been examined in slices of the guinea-pig olfactory cortex *in vitro*.

2 Concentrations of pentobarbitone, ether, methoxyflurane, trichloroethylene and alphaxalone that are known to depress synaptic transmission in the prepiriform cortex also depressed the sensitivity of prepiriform neurones to L-glutamate.

3 Halothane, in concentrations that depress synaptic transmission (< 1%) did not alter the sensitivity of neurones to glutamate. Higher concentrations (> 1%) produced a dose-related depression of the glutamate sensitivity of neurones.

4 All four volatile anaesthetics tested caused some cells to alter their glutamate-evoked firing pattern to one in which the spike discharges were more closely grouped. Pentobarbitone and alphaxalone had no such effect.

5 If the sensitivity of the neurones to the endogenous excitatory transmitter is affected by anaesthetics in the same way as the glutamate-sensitivity, these results suggest that halothane depresses synaptic transmission by decreasing the amount of transmitter released from the nerve terminals, whereas the other anaesthetics depress the sensitivity of the post-synaptic membrane to the released transmitter.

## Introduction

General anaesthetics in concentrations less than, or similar to, those required for the maintenance of anaesthesia depress excitatory synaptic transmission at a number of sites within the central nervous system, including the olfactory cortex (see Richards, 1974); this suggests that depression of excitatory synaptic transmission occurs during anaesthesia, and that it may be one of the cellular events responsible for the production of the anaesthetic state. A previous series of studies on the evoked field potentials of the olfactory cortex has shown that low concentrations of anaesthetics do not affect the electrical excitability of the fibres of the lateral olfactory tract (l.o.t.) or that of neurones of the prepiriform cortex, but they do depress the excitatory post-synaptic potentials (e.p.s.ps) that occur in the neurones of the prepiriform cortex following stimulation of the l.o.t. These results imply that anaesthetics decrease the amount of transmitter released by a nerve impulse, or they depress the sensitivity of the post-synaptic membrane to the transmitter, or both. However, field potential analysis could not by itself provide sufficient evidence to determine which of these alternatives was responsible for the observed depression of the e.p.s.ps (Richards, 1972, 1973a; Richards, Russell & Smaje, 1975). A definitive answer requires that the identity of the transmitter substance be known.

Unfortunately, the identity of the transmitter released from the l.o.t. is not established. Nonetheless, nerve cells in the prepiriform cortex can be excited by a number of substances including acetylcholine and glutamate (Legge, Randić & Straughan, 1966). While these two excitants are both thought to act directly on receptors located on the post-synaptic membrane, rather than by causing the release of other excitants, they excite neurones by quite different mechanisms. The excitation that is produced by acetylcholine is characteristically slow in onset and decline and results from a decrease in the resting membrane conductance to potassium (Krnjević, Pumain & Renaud, 1971), whereas the excitation produced by glutamate is rapid in onset and decline and mimics that of the naturally occurring transmitter (see Discussion in Richards *et al.*, 1975). In this paper we have examined the actions of six general anaesthetics on the sensitivity of neurones in the prepiriform cortex to iontophoretically applied L-glutamate. Brief accounts of some of these experiments have already been published (Richards & Smaje, 1974;

Richards *et al.*, 1975). In the accompanying paper (Smaje, 1976) the actions of general anaesthetics on the sensitivity of prepiriform neurones to iontophoretically applied acetylcholine are described.

## Methods

Details of the methods of preparation, incubation, stimulation and field potential recording have already been described in detail (Yamamoto & McIlwain, 1966; Richards *et al.*, 1975). Guinea-pigs were killed by a blow on the back of the neck, the skull was opened and the brain removed. A thin tangential slice of the olfactory cortex was then made with a razor strip and a glass template. The slices so prepared had a nominal thickness of 410 µm and were incubated in a chamber (described by Doré & Richards, 1974) at 37°C. In this chamber the slice rested on a platinum grid with its cut surface bathed by artificial c.s.f. and the uncut, pial surface exposed to a humidified atmosphere of 95% $O_2$ and 5% $CO_2$. For the experiments with the steroid anaesthetic alphaxalone, a modified chamber was used which allowed both surfaces of the slice to be superfused with saline.

### *Saline solutions*

Standard saline had the following composition (mM): NaCl 134, KCl 5, $KH_2PO_4$ 1.25, $MgSO_4$ 2, $CaCl_2$ 1, $NaHCO_3$ 16 and glucose 10. High $Mg^{2+}$ saline had a similar composition but contained 10 mM $MgSO_4$ rather than 2 mM $MgSO_4$. No osmotic compensation was made. The solutions were saturated with a mixture of 95% $O_2$ and 5% $CO_2$ before use, and had a pH of 7.2–7.4.

### *Anaesthetics*

The following anaesthetics were used: sodium pentobarbitone (Abbott Laboratories); diethyl ether (Macfarlan Smith), trichloroethylene (BDH 'Aristar' grade); halothane (redistilled from Fluothane, ICI Ltd.) and alphaxalone (Glaxo Laboratories). Sodium pentobarbitone was dissolved in the bathing saline solutions before being applied to the tissue. No pH correction was required as its concentration was very low (0.05–0.4 mM) in relation to that of the bicarbonate buffer (16 mM). The volatile anaesthetics were applied in the gas phase mixed with the stream of 95% $O_2$ and 5% $CO_2$ that superfused the upper surface of the slice. The exact concentration administered was estimated automatically by an on-line gas chromatograph (see Richards, 1973a; Richards *et al.*, 1975). Because of its highly hydrophobic nature, alphaxalone was applied as the steroid component of phospholipid vesicles (liposomes) suspended in the saline solution (see Richards & Hesketh, 1975); standard saline containing liposomes without alphaxalone was administered as a control.

### *Iontophoresis*

Conventional five-barrelled electrodes with tip diameters of 3–8 µm were used; three barrels contained 3.3 M NaCl and two 0.5 M Na L-glutamate (BDH Ltd.) (pH 7.1–7.3). One NaCl-filled barrel was used to record the activity evoked by the ejection of glutamate or the electrical stimulation of the l.o.t. (Richards & Sercombe, 1968), while another served to balance out the net currents through the electrode (Salmoiraghi & Steiner, 1963). This barrel could also be used to check for artifacts caused by the passage of current. Diffusion of glutamate from the electrode tip was prevented by retaining currents (electrode tip positive, 10–20 nA; del Castillo & Katz, 1955).

Neurones in brain tissue slices are not generally spontaneously active so single units were isolated during excitation by continuous ejection of glutamate (electrode tip negative, 10–50 nA). Thereafter, glutamate was ejected from one barrel in pulses according to a fixed schedule; the pulse duration and current remained constant throughout each trial. The most useful schedule comprised an ejection period of 10 s followed by a retention period of 20 seconds. A fixed ejection schedule is desirable as the successive pulses are alike and a constant amount of drug is given at each application.

### *Recording*

The evoked units were recorded through one NaCl-filled barrel of the iontophoretic pipette; the indifferent electrode was placed in the saline that bathed the lower surface of the slice. The recording barrel was coupled to a voltage follower or preamplifier through Ag-AgCl wires which were connected in turn to an oscilloscope and FM tape recorder. The overall flat band width of the recording system extended from 3 Hz to beyond 2.5 kHz. During the experiment the spike activity was amplified and filtered (pass band 300 Hz–10 kHz, centre frequency 2.5 kHz) and then fed into a discriminator counter which registered only those spikes whose amplitude fell between two adjustable voltage levels. The noise level of the recording system was approximately 30 µV peak-to-peak. Satisfactory discrimination of the spike discharges was generally achieved when the amplitude of a spike was greater than 100 µV.

### *Protocol*

All experiments were restricted to the prepiriform area of the olfactory cortex, i.e. the area that lies between the lateral edge of the l.o.t. and the medial border of

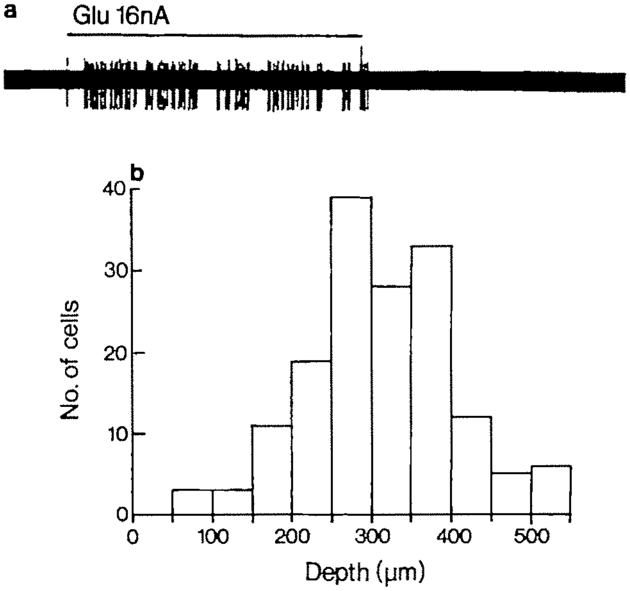


**Figure 1** Characteristics of glutamate-sensitive neurones in the prepiriform cortex. (a) An original record of a neurone responding to iontophoretically applied glutamate. This cell was located 430 μm below the pia and was not spontaneously active. Note that cell discharge starts within 600 ms of applying the glutamate and there is little after-discharge. Calibration: horizontal bar 10 seconds. (b) The depth distribution of glutamate-sensitive cells in the prepiriform cortex. These cells were not spontaneously active but could be excited by stimulation of the l.o.t.

the rhinal fissure. The small pyramidal cells that comprise layer II of this area receive a massive projection from the fibres of the l.o.t. as do the deep pyramidal cells (see Shepherd, 1974). About half the cells located by application of glutamate were also tested to see whether l.o.t. stimulation excited them; in all cases it did so. It was impossible to apply this test when synaptic transmission was blocked by high $Mg^{2+}$ saline.

If the responses of a unit to fixed pulses of glutamate were consistent for four or more minutes, the anaesthetic was introduced and the responses were followed for up to 30 min before the anaesthetic was removed and the unit allowed to recover from the anaesthetic. Before exposing a preparation to high $Mg^{2+}$ saline, it was first incubated in standard saline. Then, while the evoked field potentials were monitored, the preparation was exposed to high $Mg^{2+}$ saline. Synaptic transmission was rapidly blocked but to ensure total synaptic blockade the experiment was performed only when the evoked population e.p.s.p. was less than 5% of its initial size.

### *Data analysis*

The total number of spikes generated by a single unit in response to a given pulse of glutamate was taken as an index of a cell's sensitivity to glutamate (see Discussion section). The spike activity from representative portions of the data recorded on magnetic tape was subjected to interspike interval analysis using the special purpose computer described by Lewin (1974). A minimum of 250 spikes was used to compile each interval histogram; records obtained from cells which had a very slow firing rate during exposure to anaesthetic were generally unsuitable for interval analysis.

## Results

### *Excitation of prepiriform neurones by* L*-glutamate*

Neurones in the isolated olfactory cortex were normally electrically silent. Neuronal discharge began within 100–1000 ms of the iontophoretic application of glutamate (2–80 nA), continued as long as the glutamate was being applied and stopped within 500 ms of the ending of the application (see Figure 1). These characteristics of the glutamate-sensitivity of prepiriform neurones are similar to those of neurones in other areas of the brain (Phillis, 1970) including the cat piriform cortex *in vivo* (Legge *et al.*, 1966).

In a series of 22 experiments, 159 cells were located by iontophoretic application of L-glutamate. All of these cells could also be made to discharge in response to electrical stimulation of the l.o.t.; 75% were located 200–400 μm below the pial surface (Figure 1). This is the depth at which the dense layer of small pyramidal cells is found (layer II, see Lohman, 1963; Price, 1973).

*Effects of anaesthetics on glutamate-sensitivity in standard saline*

A total of 104 cells were studied in 71 preparations of the olfactory cortex; 76 of these cells (50 preparations) were examined in standard saline, the remainder were examined in high $Mg^{2+}$ saline. These cells were stimulated by amounts of glutamate sufficient to evoke a steady rate of firing of about 30–40 spikes per 10 s ejection period (mean 36 spikes per 10 s application; range 10–150 spikes per 10 seconds). In standard saline this firing rate was achieved with currents of 2–80 nA. The amplitude of the spikes found suitable for study with anaesthetics ranged from 100–500 μV (mean 180 μV).

*Pentobarbitone.* Twelve cells were tested for changes in their sensitivity to glutamate before, during and after exposure to concentrations of pentobarbitone within the range 0.05–0.3 mM. At 0.05 mM pentobarbitone depressed the glutamate-sensitivity of 1 cell of 6 tested, but the higher concentrations used (0.1–0.3 mM) consistently depressed the glutamate-sensitivity of all the neurones that were tested. The depression developed within 3–4 min of exposing the tissue to a saline solution containing the drug and was dose-related and reversible (Figure 2). The rate at which the neurones recovered their normal sensitivity to glutamate depended on the duration of the exposure to pentobarbitone and on the concentration applied.

*Alphaxalone.* The glutamate-sensitivity of all 11 cells studied was depressed by alphaxalone. Of these 11 cells, 7 had their glutamate-evoked activity totally suppressed and the remaining 4 cells had their sensitivity to glutamate reduced by 60–90% when the tissue was exposed to a suspension of liposomes containing alphaxalone (50 μM per 1 of saline). The onset of the depression was characteristically abrupt and occurred within 1–6 min of exposing the tissues to alphaxalone (Figure 3). When the liposomes containing alphaxalone were replaced by pure phospholipid vesicles, the cells recovered their sensitivity to glutamate, although full recovery was often protracted. Phospholipid vesicles without alphaxalone had no depressant action.

*Ether and methoxyflurane.* A total of 28 cells were tested with these anaesthetics, 11 with ether and 17 with methoxyflurane. Both anaesthetics caused a dose-related depression of the glutamate-evoked activity of neurones in the prepiriform cortex (see Figure 5a,b and Figure 8 of Richards *et al.*, 1975).

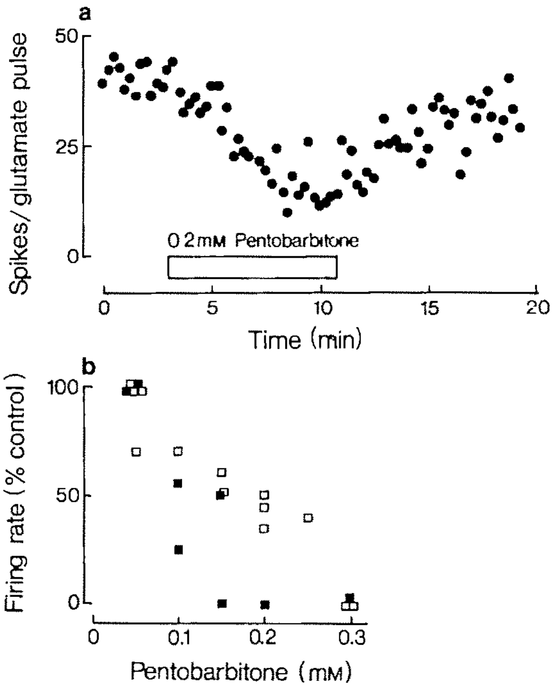


**Figure 2** The action of pentobarbitone on the sensitivity of cells in the prepiriform cortex to glutamate. (a) Shows the time course of the action of 0.2 mM pentobarbitone on the glutamate-sensitivity of a cell 210 μm deep in the prepiriform cortex. The cell was excited by a 12 nA pulse of glutamate of 10 s duration every 30 seconds. In this and subsequent figures the ordinate is the total number of spikes generated by each pulse of glutamate. (b) The relationship between the glutamate sensitivity of prepiriform neurones and the concentration of pentobarbitone applied to them. Each point represents the glutamate-evoked firing rate of a cell expressed as a percentage of the firing rate in the absence of anaesthetic. The determinations were made after equilibration with anaesthetic. (□) Experiments conducted in standard saline; (■) experiments conducted in high $Mg^{2+}$ saline.

*Trichloroethylene.* Low concentrations of trichloroethylene (0.05–0.3%) caused a dose-related depression of the glutamate-evoked activity of all 13 cells tested (Figures 4a, 5c). However, increasing the trichloroethylene concentration above 0.3% did not cause a further decrease in the responsiveness to glutamate but caused an apparent reversal of this trend until at 0.7–1% trichloroethylene the firing rate

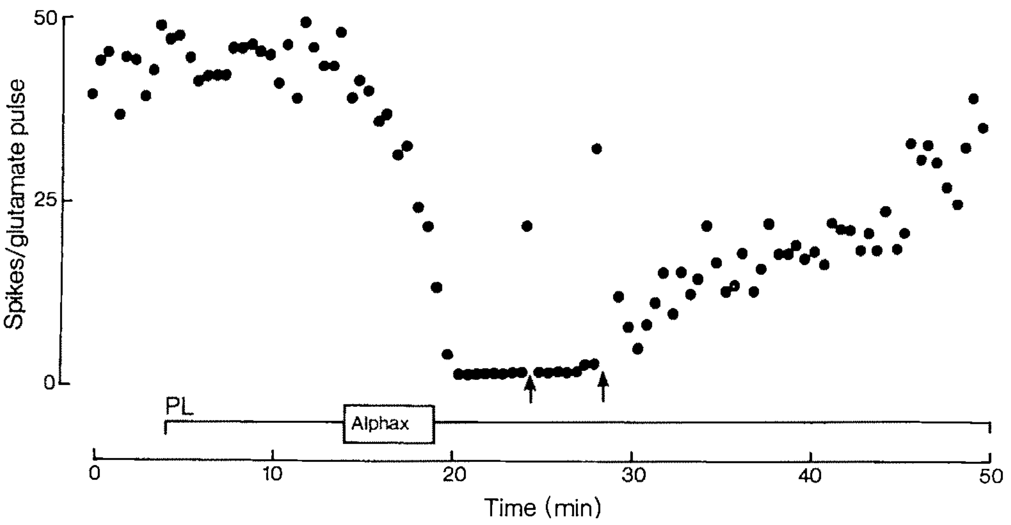


**Figure 3** The action of 50 μM alphaxalone on the glutamate-sensitivity of a neurone in the prepiriform cortex. This cell was 300 μm deep and was excited by a 16 nA pulse of glutamate of 10 s duration every 30 seconds. Abbreviations: PL, suspension of phospholipid liposomes was superfused as a control. Alphax, liposomes containing alphaxalone replaced the phospholipid liposomes. The arrows indicate 32 nA pulses of glutamate which were used to check that contact with the cell had not been lost.

equalled or exceeded that recorded in the absence of anaesthetic (3 cells). Increasing the trichloroethylene concentration still further to 1.5% resulted in the complete loss of activity.

These effects were reversible and were consistent with the idea that trichloroethylene has two actions, a depressant action on the glutamate-sensitivity and a direct stimulatory effect on the cell membrane. Evidence in favour of a direct stimulatory action was obtained from 3 cells which were exposed to 0.65–0.9% trichloroethylene; during exposure to such high concentrations all three cells began to discharge spontaneously during the periods between applications of glutamate and the spike amplitude was reduced by 10–50%.

*Halothane.* Unlike the other anaesthetics studied, halothane had no obvious depressant action on the glutamate-sensitivity of neurones in the prepiriform cortex even at concentrations known to depress synaptic transmission by 50% or more (up to 1%) (Richards, 1973a). This result was found with all 14 cells studied. One example is shown in Figure 4b. Above 1.2%, however, a depressant effect was seen which was dose-related and reversible (10 cells; Figure 5d). As with trichloroethylene, exposure to concentrations of halothane greater than 1.2% caused 3 cells (out of 10) to fire spontaneously between the iontophoretic pulses of glutamate; under these conditions the spike amplitude was reduced.

### *Effects of anaesthetics on glutamate-sensitivity in high $Mg^{2+}$ saline*

Glutamate is able to diffuse at least 300 μm through cortical tissue (Herz, Zieglgansberger & Färber, 1969) and will therefore excite a number of cells in addition to the one under observation. It is already known that anaesthetics depress synaptic transmission in the cortex (see Richards, 1974; Richards *et al.*, 1975). Therefore, the depression of the glutamate-evoked activity of a cell that results from exposure to anaesthetic might not be due to a direct effect on the postsynaptic membrane of the cell but to a depressant action on synapses of adjacent cells which could modify the activity of the cell under observation via intracortical connexions. This possibility could be excluded if similar results were obtained under circumstances that did not permit synaptic transmission. Increasing the level of $Mg^{2+}$ in the bathing medium depresses synaptic transmission between the l.o.t. and the cells of the prepiriform cortex until at 10–13 mM $Mg^{2+}$ transmission is substantially blocked (Richards & Sercombe, 1970). The same is presumably true for other synaptic contacts within the slice. Accordingly, a proportion of the experiments were conducted in high $Mg^{2+}$ saline.

The glutamate ejecting current was increased in 10 mM $Mg^{2+}$ saline in order to keep the control rates of firing comparable to those in standard saline (*ca* 35 spikes per 10 s glutamate pulse). The mean current

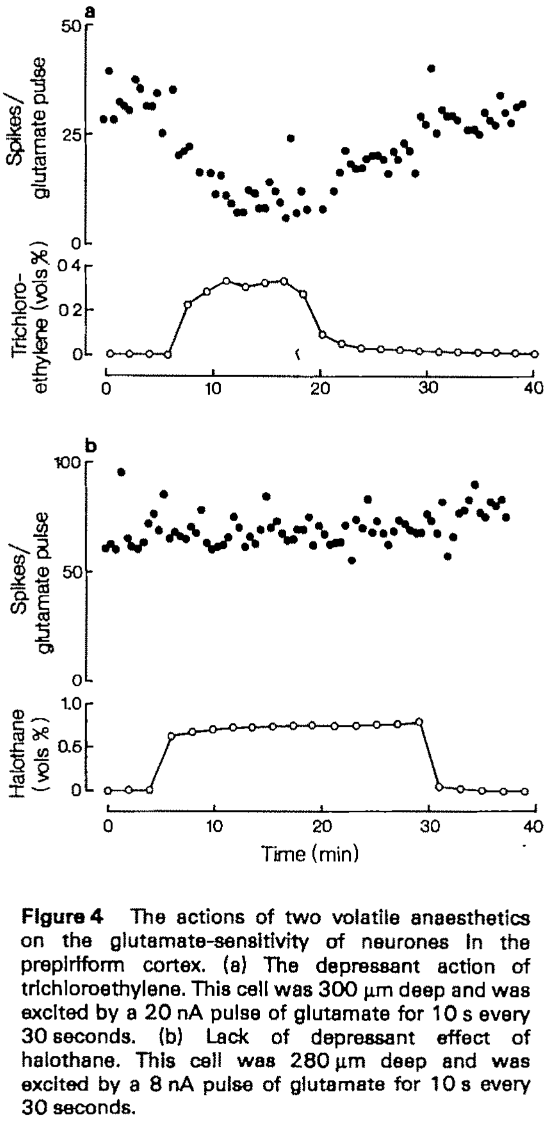


**Figure 4** The actions of two volatile anaesthetics on the glutamate-sensitivity of neurones in the prepiriform cortex. (a) The depressant action of trichloroethylene. This cell was 300 μm deep and was excited by a 20 nA pulse of glutamate for 10 s every 30 seconds. (b) Lack of depressant effect of halothane. This cell was 280 μm deep and was excited by a 8 nA pulse of glutamate for 10 s every 30 seconds.

required was 39 nA compared to a mean of 25 nA required to give comparable firing rates in 2 mM $Mg^{2+}$ saline. This increase in current reflects the increase in the threshold depolarization required for spike initiation which is known to occur when $Mg^{2+}$ concentrations are elevated (Kelly, Krnjević & Somjen, 1969; Richards & Sercombe, 1970). Nonetheless, anaesthetics depressed the sensitivity of neurones in the prepiriform cortex to iontophoretically applied glutamate in high $Mg^{2+}$ saline. This result was obtained with all five anaesthetics so examined (alphaxalone was not tested): pentobarbitone 0.05–0.3 mM, 6 units; ether 1.2–6%, 5 units; methoxyflurane 0.04–0.35%, 6 units; trichloroethylene 0.09–0.42%, 6 units; halothane 1.4–5%, 2 units (but no depression was observed at concentrations below 1%–5 units).

The actions of pentobarbitone and the volatile anaesthetics on the glutamate-sensitivity of neurones in the prepiriform cortex is summarized in Figure 2b and Figure 5 for experiments in standard and high $Mg^{2+}$ saline. It is clear from these graphs that the depressant action of anaesthetics on glutamate-sensitivity was not reduced under conditions of synaptic blockade.

### *Interspike interval distribution*

Neurones in the prepiriform cortex excited by glutamate had a variable temporal pattern of impulse generation. About 20% of the glutamate-excited units displayed a regular train of impulses; in the remainder, the impulses were grouped. Interspike interval analysis showed a single broad peak with a bias towards short intervals; the modal interval ranged from 10–200 milliseconds.

Exposure to pentobarbitone caused an increase in the modal interval commensurate with the fall in mean firing frequency (9 cells). Analysis of the four experiments with alphaxalone, in which this anaesthetic did not cause a total suppression of the glutamate responses, yielded similar results.

All the volatile anaesthetics caused a decrease in the modal interval of the interspike interval histogram in a proportion of the cells studied, despite the ability of three of them (ether, methoxyflurane and trichloroethylene) to depress the firing rate. The decrease in the modal interval reflected a tendency for the spikes to occur in tight groups or bursts; within a burst of spikes the modal intervals ranged from 4–25 milliseconds. Reducing the discharge rate merely by lowering the glutamate ejection current was never accompanied by such a change (Figure 6). Increasing the concentration of anaesthetic usually caused the grouping of impulses to become tighter. For example, one neurone exposed to 0.1% trichloroethylene fired in bursts of 2–6 spikes with a modal interval of 22.5 ms; on increasing the concentration to 0.2% the spikes then occurred in pairs or triplets with a shorter modal interval of 7.5 milliseconds. Not all cells showed changes in their firing pattern even when they were exposed to high concentrations of anaesthetic. The proportion of cells showing burst firing during exposure to anaesthetic was as follows: ether 4 of 6 cells that could be satisfactorily analysed; methoxyflurane 3/6; trichloroethylene 6/8; halothane 4/7. In the case of methoxyflurane, however, three additional units fired in bursts of spikes at the onset of exposure to anaesthetic but not while a steady concentration was maintained. This tendency for volatile anaesthetics to induce cells to fire in groups of spikes was found even in the presence of 10 mM $Mg^{2+}$; ether

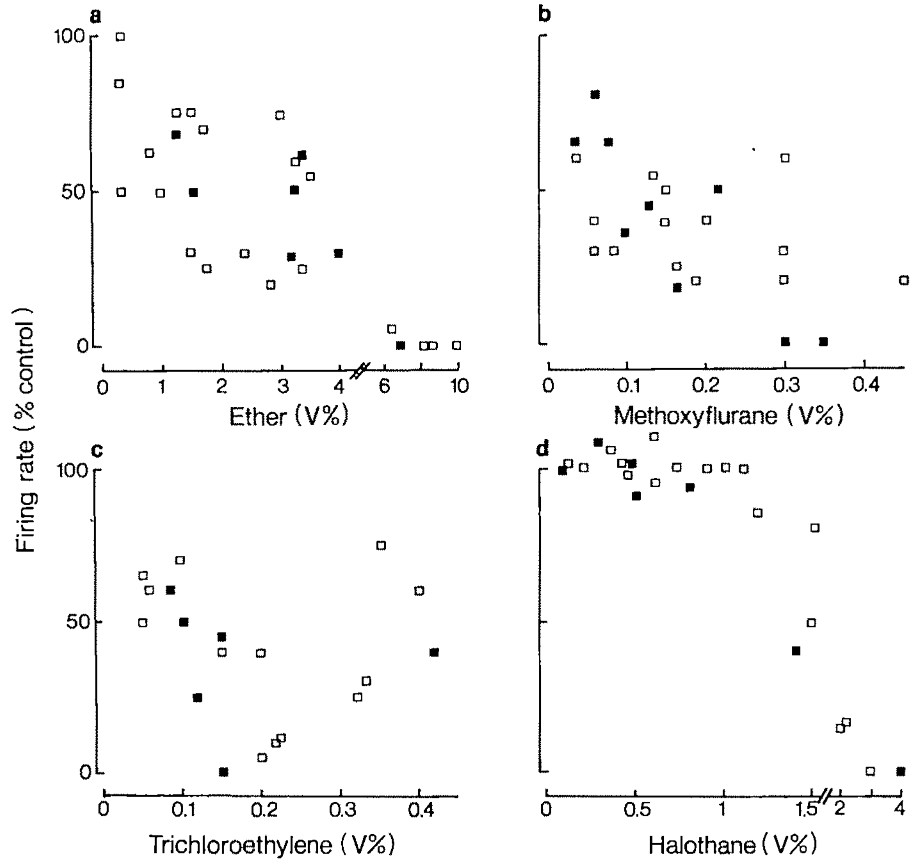


**Figure 5** Summary of the relationship between the glutamate-sensitivity of neurones in the prepiriform cortex and the concentrations of volatile anaesthetics to which they were exposed: (□) experiment conducted in standard saline; (■) experiment conducted in high $Mg^{2+}$ saline. Each symbol shows the firing rate of a cell during exposure to anaesthetic expressed as a percentage of the firing rate before exposure to anaesthetic. Some cells were exposed to two or three concentrations of anaesthetic.

0/2 cells; methoxyflurane 1/5; trichloroethylene 6/7; halothane 1/5.

### *Action of anaesthetics on the glutamate-sensitivity of cells in the guinea-pig neocortex*

To test the general validity of our results, we also examined the effects of two general anaesthetics, halothane and methoxyflurane, on the sensitivity of neocortical cells to iontophoretically applied glutamate.

A total of 14 cells were examined in 7 coronal slices of neocortex maintained *in vitro*. All 5 cells exposed to 0.03–0.3% methoxyflurane showed a dose-related depression of glutamate-evoked activity. By contrast, none of the 6 cells exposed to halothane (0.23–1.2%) showed a depression of glutamate-evoked activity at concentrations below 0.9%. Two cells showed a depression in sensitivity to glutamate when exposed to halothane concentrations between 0.9 and 1.2%.

A further 3 cells were tested in high $Mg^{2+}$ saline for changes in glutamate sensitivity during exposure to methoxyflurane. The glutamate-evoked activity of all 3 cells was depressed in a dose-related manner by 0.08–0.2% methoxyflurane.

None of the neocortical cells exposed to methoxyflurane showed any increased tendency to fire in groups of spikes whereas 3 of the 6 cells exposed to halothane did so.

## Discussion

The interpretation of our experiments rests on the assumption that the firing rate of a cell in response to a

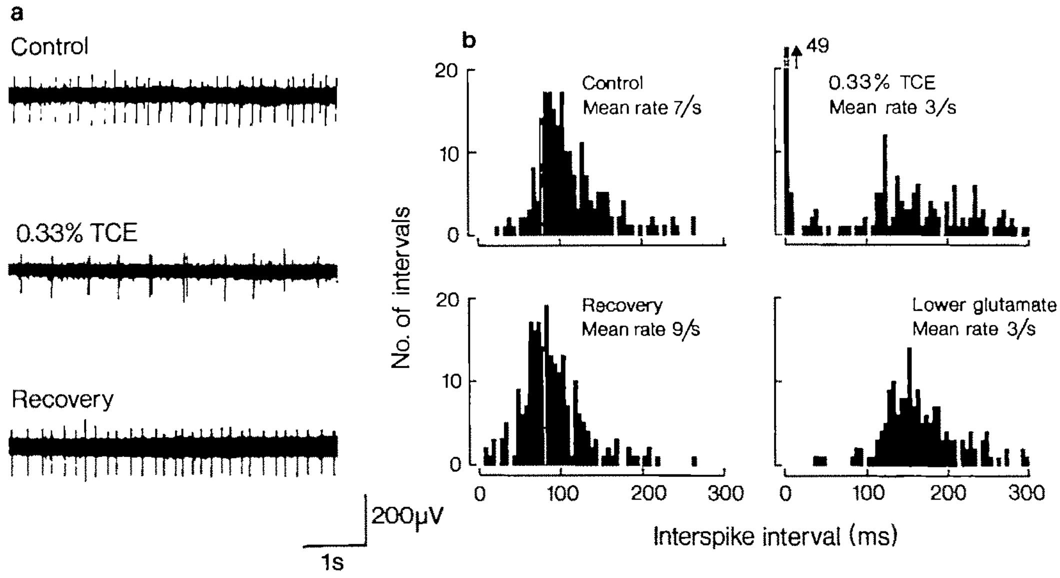


**Figure 6** Changes in the interspike interval distribution caused by exposure to trichloroethylene (TCE). (a) Filmed record of a cell firing in response to a 38 nA, 10 s pulse of glutamate in the presence and absence of trichloroethylene. This cell was 300 μm deep. Note that the cell discharges tend to occur as pairs of spikes in the presence of trichloroethylene. (b) Interspike interval histograms taken from an extended record of the same cell. Each histogram was compiled from the intervals between 250 spikes. Note the increase in the proportion of short intervals which reflects the tendency for this cell to fire in pairs of spikes during exposure to trichloroethylene. This effect could not be mimicked by reducing the glutamate excitation of the cell (30 nA).

given pulse of glutamate reflects the sensitivity of that cell to glutamate. This will only be true if there is no change in the coupling between the glutamate-induced depolarization and the generation of the action potential, if subliminal excitation and inhibition of the cell are either constant or insignificant, and if glutamate acts directly on the post-synaptic membrane and not by causing the release of some other excitatory substance. The first of these conditions is satisfied as it has already been shown that concentrations of general anaesthetics sufficient to depress synaptic transmission by 50% or more do not increase the action potential threshold, or otherwise impair the coupling between the population e.p.s.p. and the discharge of neurones in the prepiriform cortex (Richards, 1972, 1973a and unpublished data; Richards *et al.*, 1975). As the results of experiments performed on preparations bathed in standard saline were similar to those obtained from preparations in which synaptic transmission was blocked by high $Mg^{2+}$ saline (see Results section) subliminal excitation and inhibition cannot have influenced our results. Furthermore, these experiments also exclude the possibility that glutamate causes the release of some other substances from the nerve terminals as transmitter release is inhibited by high $Mg^{2+}$ saline (Richards & Sercombe, 1970; Rubin, 1970).

The reasons for choosing glutamate as an analogue for the unknown endogenous transmitter have been set out in detail elsewhere (Richards *et al.*, 1975) and may be summarized by saying that, like the transmitter itself, glutamate excites nerve cells by a sodium-dependent mechanism following its interaction with a specific receptor site. There is even some circumstantial evidence to suggest that glutamate itself may be the transmitter released from the l.o.t. nerve terminals (see Bradford & Richards, 1976). Although we confined our experiments to neurones in the prepiriform cortex, chiefly those of layer II, which are innervated by the l.o.t., it is possible that our results reflect the properties of receptors other than those responsible for synaptic transmission between the l.o.t. and the pyramidal cells of the prepiriform cortex.

All six anaesthetics that were studied depressed the firing rate of cortical neurones to iontophoretically-applied L-glutamate. For the reasons discussed earlier, these results suggest that general anaesthetics depress the sensitivity of the post-synaptic membrane itself to glutamate. As anaesthetics do not alter the coupling between the population e.p.s.p. and the discharge of the pyramidal cells, this depression is not the result of a generalized increase in the resting membrane permeability to potassium or chloride such as that seen following the iontophoretic application of 2, 4 dinitrophenol to cortical neurones (Godfraind, Krnjević & Pumain, 1970). It is more likely that this depression could reflect a decrease in the affinity of the receptor for glutamate, or a decrease in the efficacy of glutamate due to a change in receptor conformation, or a decrease in the ability of the ionophore linked to the glutamate receptor to admit ions across the membrane. At present there is no evidence which would allow us to distinguish between these possibilities.

The results summarized in Figures 2b and 5 and Table 1 show that pentobarbitone, ether, methoxyflurane and trichloroethylene all produced a dose-dependent depression of the glutamate-sensitivity of neurones within the concentration range required to depress synaptic transmission between the fibres of the l.o.t. and the neurones of the prepiriform cortex. Similarly, alphaxalone (50 μM) also depressed both sensitivity of neurones to glutamate and synaptic transmission. While our results do not exclude the possibility that these anaesthetics may depress the release of transmitter, they are consistent with the view that all five anaesthetics depress synaptic transmission, at least in part, by depressing the sensitivity of the post-synaptic membrane to endogenously released transmitter.

In contrast to the other anaesthetics studied, halothane did not depress the glutamate-sensitivity of cortical neurones until relatively high concentrations were reached (> 1%), whereas synaptic transmission became depressed at much lower concentrations (> 0.4%) (Richards, 1973a). Thus, with halothane there was little parallelism between the depression of synaptic transmission and the depression of glutamate-sensitivity. It is possible that halothane does not depress the glutamate-sensitivity of the cortical neurones because it has both a direct stimulatory action on the post-synaptic neurones as well as a depressant action on their sensitivity to glutamate. This seems unlikely as halothane does not cause the neurones to fire spontaneously until high concentrations are reached and at these concentrations (> 1.2%) their glutamate-sensitivity is depressed. Alternatively, low concentrations of halothane (< 1%) may depress synaptic transmission by reducing the output of transmitter from the nerve terminals.

The glutamate-sensitivity of neurones *in vitro* preparations of neocortex was depressed by low concentrations of methoxyflurane, but not by low concentrations of halothane, so it appears that our results do not merely reflect properties of glutamate receptors that are peculiar to the neurones of the prepiriform cortex. Furthermore, Ransom & Barker (1975) have found that the glutamate-sensitivity of spinal neurones grown in tissue culture is also depressed by pentobarbitone.

As changes in the responsiveness of a neurone to glutamate that are caused by anaesthetics *in vivo* may not only reflect changes in the sensitivity of that neurone to glutamate but also changes in background activity it is not easy to compare our results directly with those obtained in intact animals. However, there is a measure of agreement between our results and those of certain experiments conducted *in vivo*. For example, Crawford & Curtis (1966) and Johnson, Roberts & Straughan (1966) found that systemic administration of barbiturates depressed the sensitivity of cortical neurones to iontophoretically-applied glutamate and DL-homocysteic acid. Crawford (1970), working with the cerveau isolé preparation of the cat, found that halothane (< 1.5%) had no effect on the response of cells in the cat pericruciate gyrus but he also reported that methoxyflurane and trichloroethylene were without effect. Catchlove, Krnjević & Maretić (1972) using anaesthetized animals and a similar protocol to Crawford's also failed to find a depressant effect of volatile

**Table 1** Comparison of the depressant effects of anaesthetics on the population e.p.s.p. and on the glutamate-sensitivity of prepiriform neurones

| *Anaesthetic* | *Concentration required for anaesthesia* | *Depression of pop e.p.s.p. amplitude** | *Depression of glutamate firing rate†* |
|---|---|---|---|
| Pentobarbitone | 0.2 mM | 25–50% | 50–100% |
| Alphaxalone | 50 μM | 25–100% | 60–100% |
| Ether | 3.0% | 25–50% | 25–80% |
| Methoxyflurane | 0.25% | 20–40% | 40–90% |
| Trichloroethylene | 0.3% | 25–50% | 70–100% |
| Halothane | 0.9% | 25–50% | 0–10% |

* Ranges taken from the following references: Richards (1972, 1973a), Richards *et al.*, (1975), Richards & Hesketh (1975) and unpublished data.

† Ranges taken from Figures 2b and 5.

anaesthetics on the sensitivity of cortical cells to glutamate. However, in the cuneate nucleus Galindo (1969) found that pentobarbitone depressed the sensitivity of neurones to glutamate whereas halothane did not, although both anaesthetics depressed synaptic transmission between the dorsal columns and the medial lemniscus.

All the volatile agents changed the temporal firing pattern of some neurones from a more or less regular train of impulses towards a more irregular pattern in which the impulses tended to be grouped. This change in firing pattern was observed both with volatile anaesthetics that depressed the glutamate-sensitivity of neurones and with halothane which did not. It was found in preparations in which synaptic transmission was blocked with high $Mg^{2+}$ saline, and so does not depend upon the integrity of synaptic connexions but on some intrinsic property of the membrane. A similar change in the firing pattern of synaptically-evoked neurones in the prepiriform cortex has also been seen during administration of ether and trichloroethylene (Richards, 1973b; Richards *et al.*, 1975). It is reasonable to suppose that this effect is related to the burst firing of neurones seen *in vivo* during anaesthesia with volatile anaesthetics (see Goodman & Mann, 1967; Robson, 1967; Lee, 1970). This disruption of the normal temporal pattern of impulse generation may well compound the disorganizing influence of anaesthetics produced by their actions on synaptic transmission.

In conclusion, these experiments lend no support to the idea that there is a single unitary mechanism whereby anaesthetics produce their effects but rather suggest that general anaesthetics affect membrane-linked functions in a variety of ways.

J.C.S. was an M.R.C. Junior Research Fellow.

## References

BRADFORD, H.F. & RICHARDS, C.D. (1976). Specific release of endogenous glutamate from piriform cortex stimulated *in vitro*. *Brain Research*, **105**, 168–172.

DEL CASTILLO, J. & KATZ, B. (1955). On the localisation of acetylcholine receptors. *J. Physiol. Lond.*, **128**, 157–181.

CATCHLOVE, R.F.H., KRNJEVIĆ, K. & MARETIĆ, H. (1972). Similarity between effects of general anaesthetics and dinitrophenol on cortical neurones. *Can. J. Physiol & Pharmac.*, **50**, 1111–1114.

CRAWFORD, J.M. (1970). Anaesthetic agents and the chemical sensitivity of cortical neurones. *Neuropharmac.*, **9**, 31–46.

CRAWFORD, J.M. & CURTIS, D.R. (1966). Pharmacological studies on feline Betz cells. *J. Physiol. Lond.*, **186**, 121–138.

DORÉ, C.F. & RICHARDS, C.D. (1974). An improved chamber for maintaining mammalian brain tissue slices. for electrical recording. *J. Physiol., Lond.*, **239**, 83–85P.

GALINDO, A. (1969). Effects of procaine, pentobarbital and halothane on synaptic transmission in the central nervous system. *J. Pharmac. exp. Ther.*, **169**, 185–195.

GODFRAIND, J.M., KRNJEVIĆ, K. & PUMAIN, R. (1970). Unexpected features of the action of dinitrophenol on cortical neurones. *Nature, Lond.*, **228**, 562.

GOODMAN, S.J. & MANN, P.E.G. (1967). Reticular and thalamic multiple unit activity during wakefulness, sleep and anesthesia. *Exp. Neurol.*, **19**, 11–24.

HERZ, A., ZIEGLGANSBERGER, W. & FÄRBER, G. (1969). Microelectrophoretic studies concerning the spread of glutamic acid and GABA in brain tissue. *Exp. Brain. Res.*, **9**, 221–235.

JOHNSON, E.S., ROBERTS, M.H.T. & STRAUGHAN, D.W. (1969). The responses of cortical neurones to monoamines under differing anaesthetic conditions. *J. Physiol., Lond.*, **203**, 261–280.

KELLY, J.S., KRNJEVIĆ, K. & SOMJEN, G. (1969). Divalent cations and electrical properties of cortical cells. *J. Neurobiol.*, **2**, 197–208.

KRNJEVIĆ, K., PUMAIN, R. & RENAUD, L. (1971). The mechanism of excitation by acetylcholine in the cerebral cortex. *J. Physiol., Lond.*, **215**, 247–268.

LEE, B.B. (1970). The effects of anaesthesia on the visual cortex. *PhD Thesis, Univ. London.*

LEGGE, K.F., RANDIĆ, M. & STRAUGHAN D.W. (1966). The pharmacology of neurones in the pyriform cortex. *Br. J. Pharmac.*, **26**, 87–107.

LEWIN, J.E. (1974). A neurophysiological time histogram analyser. *J. Physiol., Lond.*, **239**, 86–87P.

LOHMAN, A.H.M. (1963). The anterior olfactory lobe of the guinea-pig. A descriptive and experimental anatomical study. *Acta. Anat. (Basel)*, suppl. 49, **53**, 1–109.

PHILLIS, J.W. (1970). *The Pharmacology of Synapses.* Oxford: Pergamon.

PRICE, J.L. (1973). An autoradiographic study of complementary laminar patterns of terminations of afferent fibers to the olfactory cortex. *J. comp. Neurol.*, **150**, 87–108.

RANSOM, B.R. & BARKER, J.L. (1975). Pentobarbital modulates transmitter effects on mouse spinal neurones grown in tissue culture. *Nature, Lond.*, **254**, 703–705.

RICHARDS, C.D. (1972). On the mechanism of barbiturate anaesthesia. *J. Physiol., Lond.*, **227**, 749–767.

RICHARDS, C.D. (1973a). On the mechanism of halothane anaesthesia. *J. Physiol., Lond.*, **233**, 439–456.

RICHARDS, C.D. (1973b). Does trichloroethylene have a different mode of action from other general anaesthetics? *J. Physiol., Lond.*, **233**, 25–27P.

RICHARDS, C.D. (1974). The action of general anaesthetics on synaptic transmission within the central nervous system. Ch.6 in *Molecular Mechanisms in General Anaesthesia.* ed. Halsey, M.J., Millar, R.A. & Sutton, J.A. London: Churchill-Livingstone.

RICHARDS, C.D. & HESKETH, T.R. (1975). Implications for theories of anaesthesia of antagonism between anaesthetic and non-anaesthetic steroids. *Nature, Lond.*, **256**, 179–182.

RICHARDS, C.D., RUSSELL, W.J. & SMAJE, J.C. (1975). The action of ether and methoxyflurane on synaptic transmission in isolated preparations of the mammalian cortex, *J. Physiol, Lond.*, **248**, 121–142.

RICHARDS, C.D. & SERCOMBE, R. (1968). Electrical activity observed in guinea-pig olfactory cortex maintained *in vitro*. *J. Physiol., Lond.*, **197**, 667–683.

RICHARDS, C.D. & SERCOMBE, R. (1970). Calcium, magnesium and the electrical activity of guinea-pig olfactory cortex *in vitro*. *J. Physiol., Lond.*, **211**, 571–584.

RICHARDS, C.D. & SMAJE, J.C. (1974). The actions of halothane and pentobarbitone on the sensitivity of neurones in the guinea-pig prepiriform cortex to iontophoretically applied L-glutamate. *J. Physiol., Lond.*, **239**, 103–105P.

ROBSON, J.G. (1967). The effects of anesthetic drugs on cortical units. *Anesthesiology*, **28**, 144–154.

RUBIN, R.P. (1970). The role of calcium in the release of neurotransmitter substances and hormones. *Pharmac. Rev.*, **22**, 389–428.

SALMOIRAGHI, G.C. & STEINER, F.A. (1963). Acetylcholine sensitivity of cat's medullary neurons. *J. Neurophysiol.*, **26**, 581–597.

SHEPHERD, G.M. (1974). *The Synaptic Organisation of the Brain*. Oxford: Oxford University Press.

SMAJE, J.C. (1976). General anaesthetics and the acetylcholine sensitivity of cortical neurones. *Br. J. Pharmac.*, **58**, 359–366.

YAMAMOTO, C. & McILWAIN, H. (1966). Electrical activities in thin sections from the mammalian brain maintained in chemically-defined media *in vitro*. *J. Neurochem.*, **13**, 1333–1343.

*(Received March 22, 1976)*

*Br. J. Pharmac.* (1976), **58**, 359–366

# GENERAL ANAESTHETICS AND THE ACETYLCHOLINE-SENSITIVITY OF CORTICAL NEURONES

J.C. SMAJE
National Institute for Medical Research, Mill Hill, London NW7 1AA

1 The effects of general anaesthetics on neuronal responses to iontophoretically-applied acetylcholine have been examined in slices of guinea-pig olfactory cortex maintained *in vitro*.

2 Acetylcholine excited 61% of the prepiriform neurones tested. The excitation was blocked by atropine, but not by dihydro-$\beta$-erythroidine or gallamine.

3 Alphaxalone reversibly depressed the acetylcholine-sensitivity of prepiriform neurones. Pentobarbitone did not consistently depress the acetylcholine sensitivity of these cells.

4 Ether, methoxyflurane, trichloroethylene and halothane caused a dose-related augmentation of acetylcholine-induced firing.

5 These results show that general anaesthetics do not necessarily depress the sensitivity of nerve cells to all excitatory substances and that different anaesthetics may affect a particular excitatory process in various ways.

## Introduction

Previous studies have shown that general anaesthetics depress excitatory synaptic transmission in isolated slices of the guinea-pig olfactory cortex (Richards, 1974), and that the sensitivity of prepiriform neurones to iontophoretically-applied L-glutamate is depressed by similar concentrations of anaesthetic (with the exception of halothane; Richards & Smaje, 1976). The properties of glutamate resemble those of the natural transmitter at synapses between the lateral olfactory tract (l.o.t.) and prepiriform pyramidal cells. Thus, depression of synaptic transmission by anaesthetics other than halothane was attributed (at least in part) to an action on the postsynaptic sensitivity of prepiriform cells to the l.o.t. transmitter substance. The question arises whether the receptivity of these neurones to other excitatory substances will be affected in the same manner, or whether the depressant action of anaesthetics is specific for the excitation produced by glutamate and the l.o.t. transmitter.

Iontophoretic application of acetylcholine to olfactory cortex neurones causes excitation that is slow in both onset and decline, having the pharmacological properties of a predominantly muscarinic process (Legge, Randić & Straughan, 1966). Muscarinic excitation of cortical neurones is thought to result from a decrease in membrane permeability to potassium ions (Krnjević, Pumain & Renaud, 1971); the actions of acetylcholine, therefore, could be quite different in nature from the rapid actions of glutamate and the l.o.t. transmitter substance, which may be mediated by an increase in the membrane permeability to sodium (Richards, Russell & Smaje, 1975; Richards & Smaje, 1976).

This paper describes the actions of six general anaesthetics on the acetylcholine-sensitivity of prepiriform neurones. It will be shown that the four volatile agents tested (ether, methoxyflurane, trichloroethylene and halothane) caused an increased sensitivity of prepiriform neurones to acetylcholine. Pentobarbitone had no consistent effect on the sensitivity of neurones in the prepiriform cortex to acetylcholine. The steroid anaesthetic alphaxalone depressed the sensitivity of these neurones to acetylcholine in a similar manner to its action on their sensitivity to glutamate.

## Methods

The methods were substantially those of the preceding paper (Richards & Smaje, 1976), with the following modifications.

### *Saline solutions*

Standard saline had the following composition (mM): NaCl 134, KCl 5, $KH_2PO_4$ 1.25, $MgSO_4$ 2, $CaCl_2$ 1,

$NaHCO_3$ 16 and glucose 10. High $Mg^{2+}$-low $Ca^{2+}$ saline had the same composition as standard but with $MgSO_4$ 10 mM and $CaCl_2$ 0.75 mM, or as standard but with $MgSO_4$ 4 mM and $CaCl_2$, 0.5 mM.

### *Drugs*

The following drugs were used, dissolved in the appropriate saline solution: atropine sulphate (BDH), dihydro-β-erythroidine hydrobromide (DHBE; kindly donated by Merck, Sharpe & Dohme), gallamine triethiodide (May & Baker), physostigmine sulphate (Wellcome).

### *Iontophoresis*

At least two barrels of the five-barrelled microelectrodes contained 3.3 M NaCl and at least one contained Na L-glutamate (0.5 M, pH 7.1–7.3; BDH). In addition, at least one barrel contained acetylcholine chloride (0.2 M, pH 4–5; Roche). The electrodes were used within 24 h of filling. Current balancing was used in all experiments, except in some in which neurones were deliberately excited by anionic current passed from one or two NaCl-filled barrels.

## Results

### *Acetylcholine-sensitivity of prepiriform neurones*

In a series of 159 prepiriform neurones in 22 preparations located at random with glutamate, 97 (61%) could also be excited by acetylcholine (10–160 nA). The effect of acetylcholine was slow in onset (usually between 2 and 120 s) and persisted for a similar period after cessation of ejection (see Figure 2a). A temporary increase in firing rate often followed the termination of acetylcholine application, and a few units did not respond at all until this time. The depth distribution and spike amplitudes of cholinoceptive cells were not significantly different from those of cells responsive only to glutamate (Smaje, 1976).

When acetylcholine was ejected against a background of continuous glutamate excitation, an inhibitory effect starting within 2 s was revealed in all but one of 22 cells so examined. The characteristic action of acetylcholine (18/22 cells) was biphasic, the initial inhibition being followed by excitation within 2 s to several minutes.

In agreement with earlier field potential studies in the olfactory cortex (see Richards & Sercombe, 1970), the muscarinic blocking agent atropine (5 μM) blocked the excitatory effect of acetylcholine (but not that of glutamate) whereas the nicotinic blocking agents DHBE and gallamine (both 10 μM) did not. The excitatory action of acetylcholine was therefore considered to be muscarinic.

Frequently, a neurone that had been sensitive to acetylcholine would cease to be excited by acetylcholine. Sensitivity could occasionally be restored if a brief pulse of glutamate was used to initiate firing, or if the acetylcholine ejecting current was increased. However, approximately one in every six experiments was abandoned because the neurone became refractory to acetylcholine during the initial few minutes of the control period. Neither excitation by glutamate nor inhibition by acetylcholine were lost in this manner.

### *Effects of anaesthetics on acetylcholine-sensitivity*

This was investigated on 61 cells in 43 preparations in standard saline. Acetylcholine was expelled with pulses of current, usually lasting 10 s and repeated every 30 s throughout an experiment. The mean number of spikes generated in response to each 10 s pulse (including after-discharge) was 85 (range 40–200 spikes), and was achieved with currents in the range 14–140 nA; mean 74 nA. Anaesthetics were delivered in concentrations known to depress excitatory synaptic transmission and glutamate sensitivity in the olfactory cortex (Richards & Smaje, 1976).

*Pentobarbitone.* Pentobarbitone (0.05–0.40 mM) had little or no effect on the responses of almost half (8/17) of the cells excited by acetylcholine. The sensitivity of the other 9 cells to acetylcholine was totally lost 2–22 min after the beginning of the exposure to pentobarbitone. This loss of sensitivity to acetylcholine was independent of the concentration of pentobarbitone applied. When a cell became unresponsive to acetylcholine, it was excited by applying glutamate to confirm that it was still near the electrode. Only three of the cells tested in this manner recovered their sensitivity to acetylcholine within 30 min of washing out the pentobarbitone.

*Alphaxalone.* Alphaxalone (50 μM) was delivered to the preparations as a component of phospholipid vesicles suspended in standard saline (Richards & Hesketh, 1975). The acetylcholine-evoked activity of all 5 neurones studied was abolished 1–6 min after the introduction of alphaxalone. Recovery was observed within 20 min in four cases, one such experiment being shown in Figure 1, but was negligible at 30 min in the fifth. Phospholipid suspension without the anaesthetic component served as the control solution in these experiments, and had no depressant effect on the acetylcholine responses of prepiriform neurones.

*Volatile anaesthetics.* Exposure to volatile agents in low to moderate concentrations caused a prompt and sustained augmentation of the acetylcholine-induced

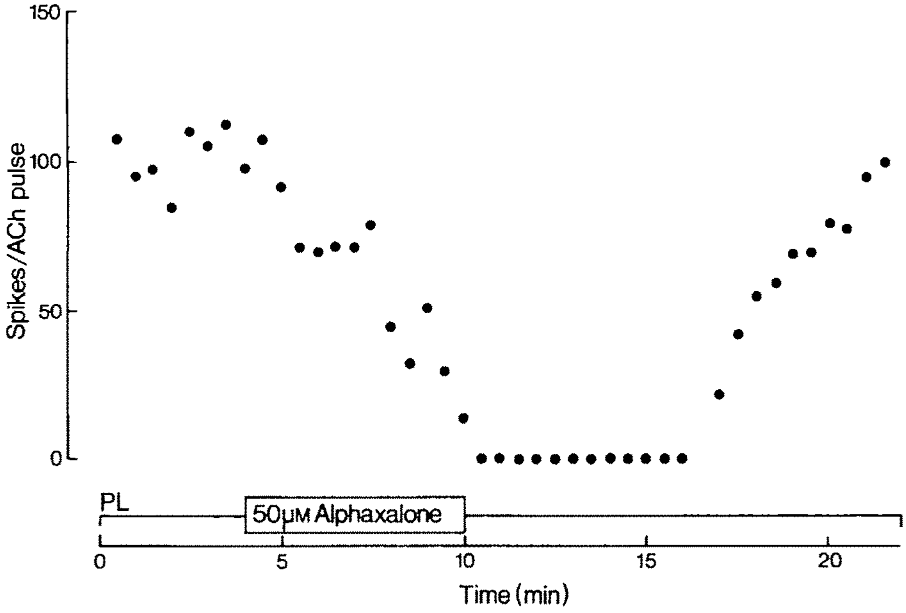


**Figure 1** The effect of alphaxalone on the acetylcholine-sensitivity of a neurone in the prepiriform cortex. This cell was 390 μm deep and was excited by a 75 nA pulse of acetylcholine for 10 s every 30 seconds. PL, phospholipid liposome suspension superfused as a control; Alphaxalone, liposomes containing alphaxalone in a 1 : 1 molar ratio with phospholipid. The final molarity of the alphaxalone in the suspension was 50 μM.

firing of all cells studied (ether 0.5–2.9%, 10 units; methoxyflurane 0.05–0.35%, 11 units; trichloroethylene 0.03–0.40%, 11 units; halothane 0.09–1.0%, 6 units). Figure 2 presents a typical experiment in which a 14 min exposure to 0.4–0.5% halothane caused the mean firing rate of the neurone to be approximately doubled. The delay in the onset of cholinergic excitation was reduced, the steady level of firing increased, and the after-discharge lengthened. Although action potentials could sometimes not be recorded in the presence of high concentrations of anaesthetic, this may have been due to depolarization block, rather than to depression of sensitivity to acetylcholine. This is illustrated in Figure 3, in which the firing frequency of a cell excited by acetylcholine rapidly dropped when the concentration of trichloroethylene was increased from 0.2 to 0.5%; the spike amplitude was considerably diminished when the cell was discharging at its maximum rate. Figure 4 summarizes the results obtained with each volatile anaesthetic (open squares): the dose-related nature of the effects is evident. Refractoriness to the excitatory action of acetylcholine never developed during the delivery of a volatile anaesthetic.

Four possible explanations for these results were considered: first, anaesthetics may have depressed inhibitory influences from adjacent neurones in synaptic contact with the cell under observation. This was excluded on the basis of experiments performed under conditions of synaptic blockade. Increase of the magnesium and reduction of the calcium content of the bathing saline combine to suppress synaptic transmission in the olfactory cortex slice, probably by reducing transmitter output, as their effects on postsynaptic threshold tend to cancel (Richards & Sercombe, 1970). Accordingly, three experiments with trichloroethylene and five with halothane were performed in preparations equilibrated with high $Mg^{2+}$-low $Ca^{2+}$ saline; the results were similar to those in standard saline (filled squares, Figure 4).

Second, a direct stimulatory action of the anaesthetics was considered. A control level of firing, without the intermediary of a chemical excitant, was provided by extracellular anionic current stimulation (range 120–240 nA, mean 180 nA; 10 s pulses every 30 s) (Krnjević & Phillis, 1963a). All the experiments were conducted in high $Mg^{2+}$-low $Ca^{2+}$ saline to prevent the release of transmitter substances from other depolarized structures. Six units were studied with trichloroethylene (0.06–0.60%) and six with halothane (0.15–1.3%). Both anaesthetics had a mild stimulatory effect in high concentration (trichloroethylene >0.4%, halothane >0.9%), presumably corresponding to that seen in previous experiments

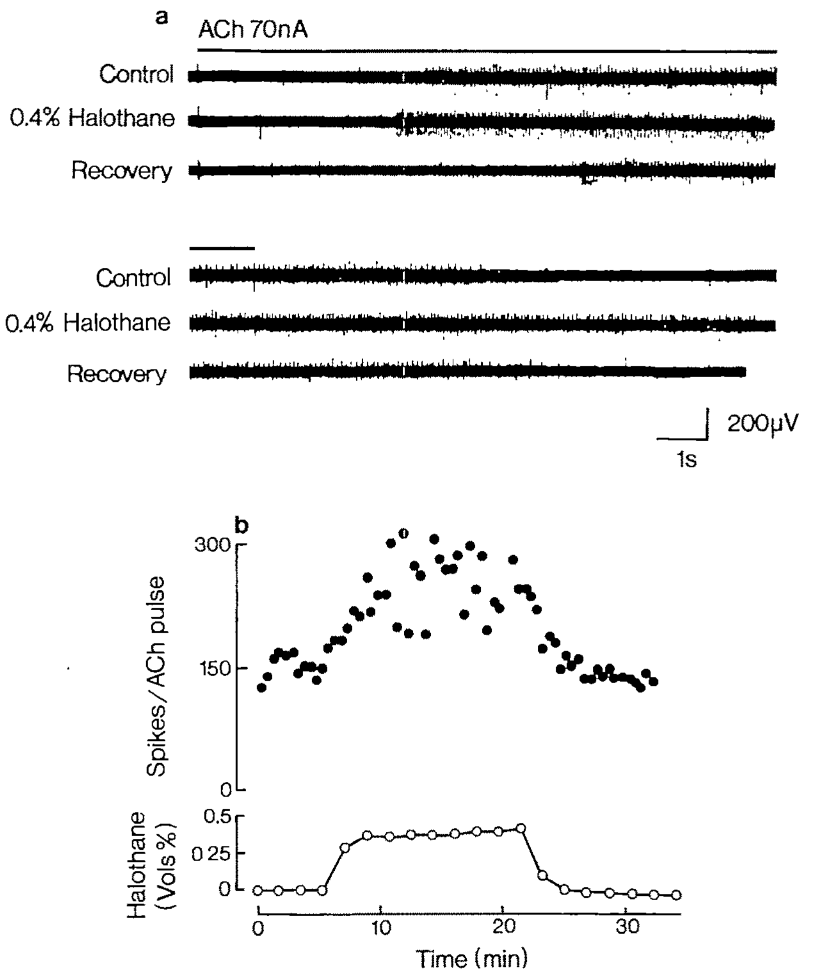


**Figure 2** The action of halothane on the sensitivity of a neurone to iontophoretically-applied acetylcholine. (a) The discharge of a neurone in response to a 15 s 70 nA pulse of acetylcholine (ACh, black bar) before, during and after exposure to halothane. The records in the two panels are continuous. This cell was 290 μm deep in the prepiriform cortex. (b) Summary of the time course of the action of halothane on the cell shown in (a).

(Richards & Smaje, 1976), but inadequate in itself to account for the acceleration of acetylcholine responses.

Third, a capacity of volatile anaesthetics to suppress cholinergic inhibition was examined in six preparations treated with atropine; cholinergic inhibition was then revealed against a background of glutamate excitation. As halothane does not depress the glutamate excitation of cortical neurones (Richards & Smaje, 1974, 1976) its action on cholinergic inhibition was studied. No significant effect on the inhibitory action of acetylcholine was observed with halothane administered in concentrations from 0.2 to 1.1% (7 units; Figure 5).

Finally, it was possible that volatile anaesthetics inhibited the hydrolysis of acetylcholine by acetylcholinesterase. This was tested in preparations whose cholinesterase activity was already abolished by treatment with physostigmine (7.8–78 μM). Difficulty was experienced in obtaining satisfactory responses to acetylcholine as refractoriness to the excitatory action of acetylcholine, or rapid depolarization block, commonly developed. As a result, only one neurone was successfully studied. The acetylcholine-induced firing rate of this cell rose during a 7 min exposure to trichloroethylene (0.2–0.35%) to over twice that in the control period; the neurone became refractory to acetylcholine at the termination of anaesthetic

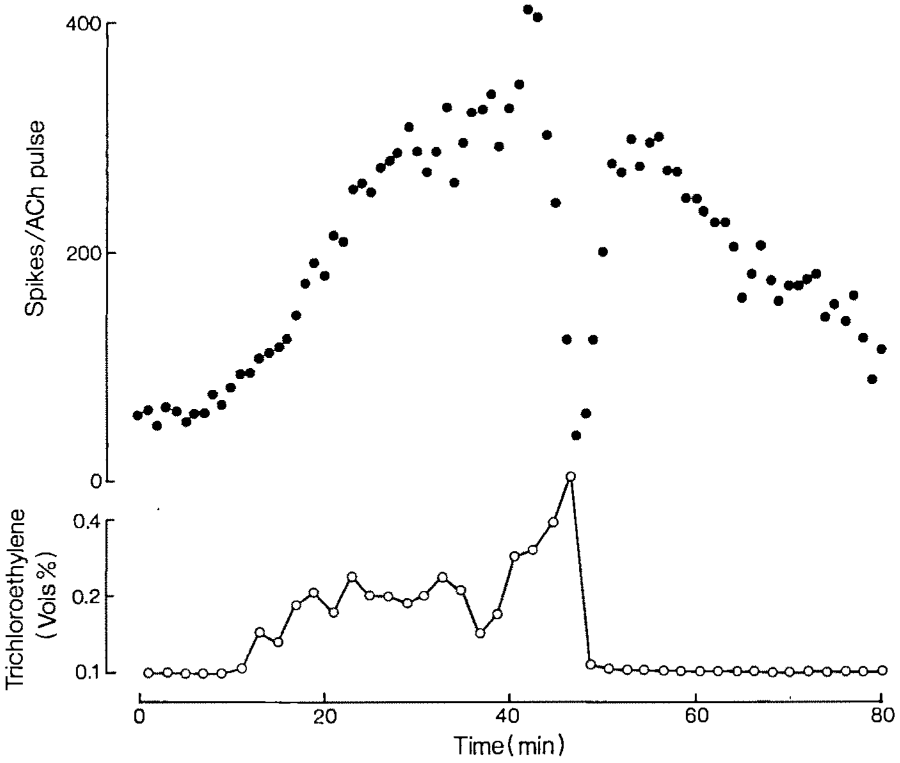


**Figure 3** The actions of trichloroethylene on the sensitivity of a neurone to iontophoretically-applied acetylcholine. This cell was 310 μm deep and was excited by a 90 nA pulse of acetylcholine for 10 s every 30 seconds. Trichloroethylene (0.2%) caused the firing rate of the cell to be quadrupled but increasing the anaesthetic to 0.5% caused a block of the responsiveness to acetylcholine. Both effects were reversed as the anaesthetic was removed.

delivery. The acceleration of acetylcholine responses cannot have been due to inhibition of acetylcholinesterase in the absence of significant activity of this enzyme.

### *Experiments with neocortical preparations*

A small number of experiments was also performed with coronal slices of the guinea-pig sensorimotor neocortex. Five neurones in separate preparations were exposed to trichloroethylene (0.07–0.25%). The responses of these cells to acetylcholine were increased during exposure to this anaesthetic. This effect was very similar to that observed in the olfactory cortex preparation.

## Discussion

The excitatory responses of isolated prepiriform neurones to acetylcholine closely resembled those seen in the cat piriform cortex *in vivo* (Legge *et al.* 1966). These authors do not mention the development of refractoriness to acetylcholine, although this does occur in the thalamus (Andersen & Curtis, 1964; McCance, Phillis & Westerman, 1968), and desensitization to acetylcholine is well known in other cholinoceptive tissues (Michelson & Zeimal, 1973). Expression of the phenomenon was modified by anaesthetics: no unit ceased responding to acetylcholine in the presence of a volatile agent, whilst several did so during exposure to pentobarbitone.

The assumption that the firing rate of a neurone in response to acetylcholine reflects the sensitivity of that cell to acetylcholine rests on several conditions. First, that there be no change in the coupling between depolarization and discharge in the presence of an anaesthetic; this has been considered in the accompanying paper (Richards & Smaje, 1976). Next, that subliminal excitation or inhibition from adjacent neurones (or from an intermediate substance that may be released) should be constant or insignificant; the

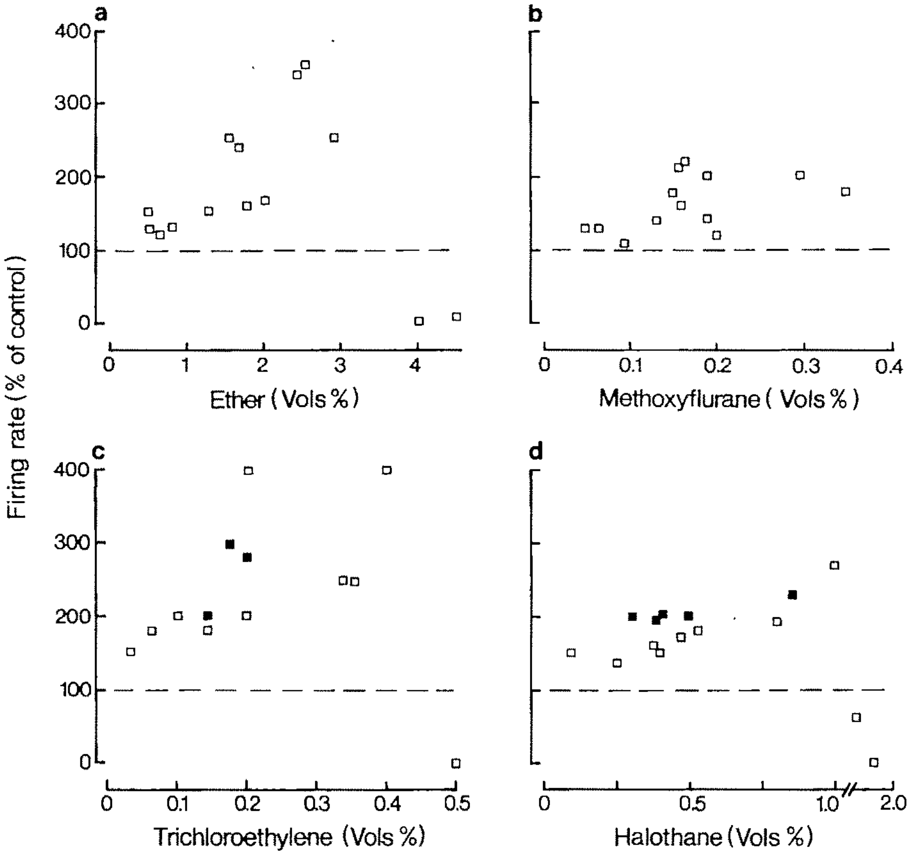


**Figure 4** Summary of the relationship between the acetylcholine responses of neurones in the prepiriform cortex and the concentrations of volatile anaesthetics applied. The results are expressed as a percentage of the acetylcholine-evoked firing rate observed in the absence of anaesthetic. (The normalized control firing rate is indicated by the horizontal dotted line). (□) Experiments in standard saline; (■) experiments in high $Mg^{2+}$- low $Ca^{2+}$ saline.

experiments with high $Mg^{2+}$-low $Ca^{2+}$ saline suggest that this was true. Furthermore, the potentiating effect of volatile anaesthetics on the acetylcholine responses of prepiriform neurones was shown not to result from direct stimulation by the anaesthetics, or from reduction in cholinergic inhibition or cholinesterase activity. The remaining possibilities are that volatile anaesthetics increase the affinity of muscarinic receptors for acetylcholine, or that they increase the efficacy of acetylcholine in depolarizing the neuronal membrane through changes in the conformation of the receptor or receptor-linked ionophore. As the excitation produced by acetylcholine is thought to result from a decrease in the permeability of the resting membrane to potassium (Krnjević *et al.* 1971), one possible effect of volatile agents could be to decrease further the resting $K^+$ conductance. If these agents also interfered with the $K^+$ conductance increase that follows the entry of sodium during an action potential, the repolarizing phase of a spike would be prolonged; this could provide an explanation for the burst firing observed in glutamate-excited neurones (Richards *et al.*, 1975; Richards & Smaje, 1976). An intracellular investigation of *Aplysia* neurones has shown various agents (including the volatile anaesthetic chloroform, but also pentobarbitone) to cause changes in $K^+$ conductance of this sort (Barker, 1975b). Intracellular studies in the mammalian brain are required to explore this possibility further.

The effects of volatile anaesthetics on the sensitivity of neurones to acetylcholine may be contrasted with their depressant action on the glutamate-sensitivity of neurones in the same region of cortex.

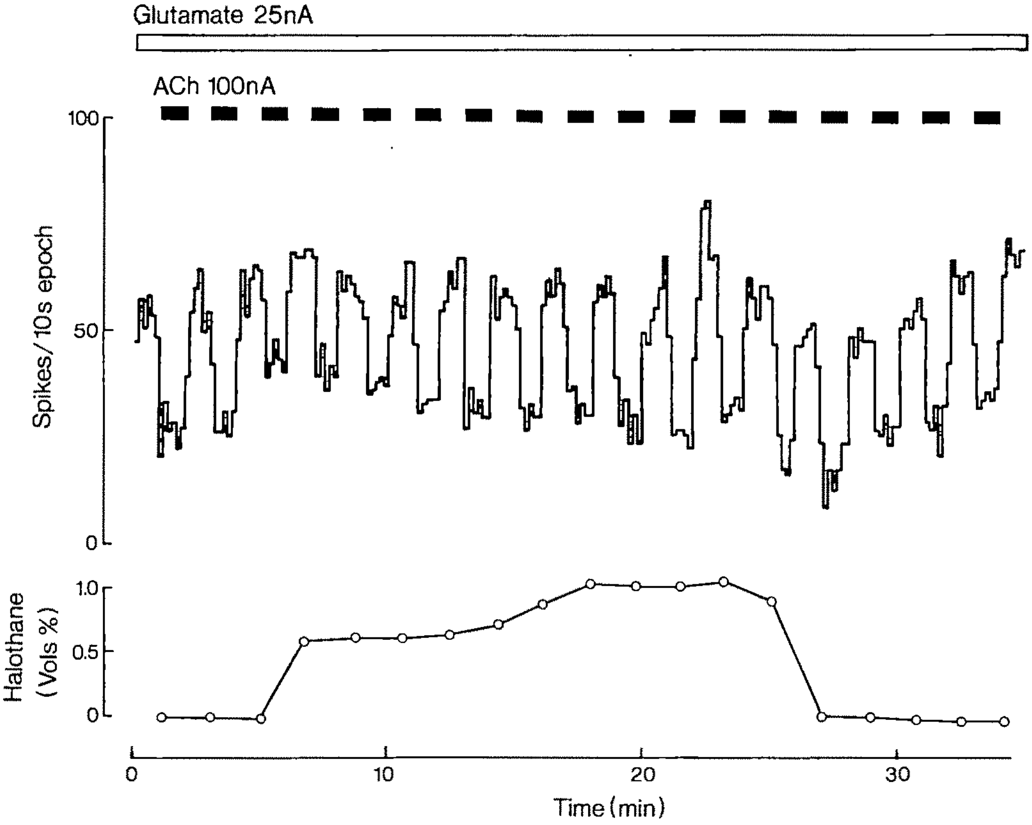


**Figure 5** The effect of halothane on the inhibitory action of acetylcholine on a neurone in the prepiriform cortex. This cell was 400 µm deep in the prepiriform cortex and was excited by a continuous release of glutamate (25 nA). The acetylcholine (ACh, 100 nA) was released for 1 min periods every 2 min (indicated by black bars). The experiment was conducted in standard saline containing atropine (0.2 mM) in order to block the excitatory actions of acetylcholine. Halothane (up to 1.1%) had no effect on the inhibitory action of acetylcholine.

Although pentobarbitone depressed the glutamate-sensitivity of neurones, it failed to depress the acetylcholine-sensitivity of those cells that did not become totally refractory to acetylcholine. Only alphaxalone reversibly depressed both the glutamate-sensitivity and the acetylcholine-sensitivity of neurones. These findings show that different anaesthetics do not necessarily share a common mode of action.

A particular advantage of *in vitro* preparations of the central nervous system is the absence of spontaneous nerve cell activity. This has made possible the study of the action of anaesthetics on the sensitivity of individual nerve cells to excitants without the variations in subliminal excitation and inhibition that are characteristic of the intact animal. The fact that such influences have been removed should be considered when the present experiments are compared to those conducted *in vivo*. Systemic administration of barbiturates (Krnjević & Phillis, 1963b; Crawford & Curtis, 1966; Crawford, 1970; Catchlove, Krnjević & Maretić, 1972) and high concentrations of volatile anaesthetics (Catchlove *et al.*, 1972) have been shown to depress the sensitivity of cortical neurones to acetylcholine in parallel with their depression of spontaneous nerve cell activity. However, when Crawford (1970) applied pentobarbitone iontophoretically he found little depressant effect on the acetylcholine-sensitivity of cortical neurones; he also showed that methoxyflurane, trichloroethylene and halothane did not depress the sensitivity of cortical neurones to acetylcholine when delivered in moderate concentrations.

To conclude, the present experiments together with those of the previous paper (Richards & Smaje, 1976) have shown that the depolarizing action of glutamate is depressed by general anaesthetics whereas that of acetylcholine was depressed by alphaxalone but not by other general anaesthetics. As muscarinic excitation of cortical neurones is thought to result from a decrease in the resting membrane permeability to potassium ions (Krnjević *et al.*, 1971) while glutamate excitation is thought to result from an increase in the sodium permeability (see Richards *et al.*, 1975), these results are consistent with the suggestion of Barker (1975a) that most general anaesthetics depress the sensitivity of the post-synaptic membrane to chemical transmitters only where the transmitters cause an increased membrane permeability to sodium.

I wish to thank Dr C.D. Richards for helpful discussions and criticism of the manuscript. The author was an M.R.C. Junior Research Fellow.

## References

ANDERSEN, P. & CURTIS, D.R. (1964). The excitation of thalamic neurones by acetylcholine. *Acta physiol. scand.*, **61**, 85–99.

BARKER, J.L. (1975a). CNS depressants: effects on post-synaptic pharmacology. *Brain Res.*, **92**, 35–55.

BARKER, J.L. (1975b). Inhibitory and excitatory effects of CNS depressants on invertebrate synapses. *Brain Res.*, **93**, 77–90.

CATCHLOVE, R.F.H., KRNJEVIĆ, K. & MARETIĆ, H. (1972). Similarity between effects of general anaesthetics and dinitrophenol on cortical neurones. *Can. J. Physiol. Pharmac.*, **50**, 1111–1114.

CRAWFORD, J.M. (1970). Anaesthetic agents and the chemical sensitivity of cortical neurones. *Neuropharmacology*, **9**, 31–46.

CRAWFORD, J.M. & CURTIS, D.R. (1966). Pharmacological studies on feline Betz cells. *J. Physiol., Lond.*, **186**, 121–138.

KRNJEVIĆ, K. & PHILLIS, J.W. (1963a). Iontophoretic studies of neurones in the mammalian cerebral cortex. *J. Physiol., Lond.*, **165**, 274–304.

KRNJEVIĆ, K. & PHILLIS, J.W. (1963b). Pharmacological properties of acetylcholine-sensitive cells in the cerebral cortex. *J. Physiol., Lond.*, **166**, 328–350.

KRNJEVIĆ, K., PUMAIN, R. & RENAUD, L. (1971). The mechanism of excitation by acetylcholine in the cerebral cortex. *J. Physiol., Lond.*, **215**, 247–268.

LEGGE, K.F., RANDIĆ, M. & STRAUGHAN, D.W. (1966). The pharmacology of neurones in the pyriform cortex. *Br. J. Pharmac.*, **26**, 87–107.

McCANCE, I., PHILLIS, J.W. & WESTERMAN, R.A. (1968). Acetylcholine-sensitivity of thalamic neurones: its relationship to synaptic transmission. *Br. J. Pharmac.*, **32**, 635–651.

MICHELSON, M.J. & ZEIMAL, E.V. (1973). *Acetylcholine. An Approach to the Molecular Mechanism of Action.* Oxford: Pergamon.

RICHARDS, C.D. (1974). The action of general anaesthetics on synaptic transmission within the central nervous system. In *Molecular Mechanisms in General Anaesthesia.* ed. Halsey, M.J., Millar, R.A. & Sutton, J.A. pp. 90–109. London: Churchill–Livingstone.

RICHARDS, C.D. & HESKETH, T.R. (1975). Implications for theories of anaesthesia of antagonism between anaesthetic and non-anaesthetic steroids. *Nature, Lond.*, **256**, 179–182.

RICHARDS, C.D., RUSSELL, W.J. & SMAJE, J.C. (1975). The action of ether and methoxyflurane on synaptic transmission in isolated preparations of the mammalian cortex. *J. Physiol., Lond.*, **248**, 121–142.

RICHARDS, C.D. & SERCOMBE, R. (1970). Calcium, magnesium and the electrical activity of guinea-pig olfactory cortex *in vitro. J. Physiol., Lond.*, **211**, 571–584.

RICHARDS, C.D. & SMAJE, J.C. (1974). The actions of halothane and pentobarbitone on the sensitivity of neurones in the guinea-pig prepiriform cortex to iontophoretically applied L-glutamate. *J. Physiol., Lond.*, **239**, 103–105P.

RICHARDS, C.D. & SMAJE, J.C. (1976). Anaesthetics depress the sensitivity of cortical neurones to L-glutamate. *Br. J. Pharmac.*, **58**, 347–357.

SMAJE, J.C. (1976). The effects of general anaesthetics on the chemical sensitivity of cortical neurones *in vitro. PhD Thesis, University of London.*

*(Received March 22, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 367–372

# MAZINDOL ANOREXIA IS MEDIATED BY ACTIVATION OF DOPAMINERGIC MECHANISMS

Z.L. KRUK & M.R. ZARRINDAST
Department of Pharmacology and Therapeutics, The London Hospital Medical College, Turner Street, London E1 2AD

**1** Anorexia in rats following injections of mazindol (0.1–8 mg/kg i.p.) could be antagonized by pretreatment with a dopamine receptor blocker (pimozide) but not by pretreatment with an $\alpha$-adrenoceptor blocker (phenoxybenzamine), a $\beta$-adrenoceptor blocker ((−)-propranolol), or a 5-hydroxytryptamine receptor blocker (methergoline).

**2** In rats with a unilateral lesion in the substantia nigra made by stereotaxic injection of 6-hydroxydopamine, mazindol caused a dose-dependent turning towards the lesioned side, indicating an indirect mechanism of action. This effect could be antagonized by pretreatment with a dopamine receptor blocker.

**3** In rats pretreated with reserpine and $\alpha$-methyl-*p*-tyrosine, mazindol did not have any motor stimulant action.

**4** *In vitro* studies with synaptosomes prepared from rat brain, indicated that mazindol blocks uptake and causes release of dopamine.

**5** It is concluded that the anorectic action of mazindol is mediated by a dopaminergic mechanism.

## Introduction

Mazindol has recently been introduced as an anorectic, and it differs from all other previously available anorectics in that it is not structurally related to amphetamine. Gogerty, Penberthy, Iorio & Trapold (1975) have described it as having weak central nervous system stimulant properties with marked anorectic activity. Gogerty *et al.* (1975) and Engstrom, Kelly & Gogerty (1975), suggest that the anorectic activity of mazindol is due to its ability to block neuronal uptake of noradrenaline. As noradrenaline is known to stimulate rather than inhibit feeding when applied intracerebrally (Booth, 1968; Slangen & Miller, 1969), while dopamine and 5-hydroxytryptamine (5-HT) inhibit feeding when applied by this route (Kruk, 1973), it was decided to examine the activity of specific receptor antagonists on the anorectic response to mazindol. In the event, dopamine receptor stimulation was indicated, and the activity of mazindol in other dopamine-sensitive animal models *in vivo* was examined. Finally, *in vitro* studies of uptake and release of dopamine using neostriatal synaptosomes were made to confirm the evidence obtained by the *in vivo* studies.

## Methods

### *Measurement of anorectic response in rats*

Groups of six adult Sprague-Dawley rats were housed in a room maintained at 20°C ± 1°C and a 12 h light, 12 h dark cycle, the light cycle starting at 07 h 00 min. The rats were trained to eat their daily food intake in a period of 4 h, and the amount eaten after 1, 2 and 4 h was recorded. Water was available at all times. All drugs were injected intraperitoneally, and the doses refer to the form listed below. Anorectic drugs were always administered at 11 h 30 min, which was 30 min before food presentation at 12 h 00 min. Pretreatment times refer to the time before the start of the feeding period. Inhibition of feeding was expressed as the percentage decrease in food eaten in the first hour following drug treatment, when compared with appropriate saline (0.9% w/v NaCl solution) control for that group on the previous day. $ED_{50}$ values (defined as the dose of drug required to decrease the food eaten by 50% in the first hour) were calculated by the method of Litchfield & Wilcoxon (1949). At least five groups of six animals were always used to determine any dose-response curve.

### *Unilateral injections of 6-hydroxydopamine*

Male rats weighing 160–180 g were anaesthetized with sodium methohexitone (Brietal, Eli Lilly) 50 mg/kg, and placed in a David Kopff stereotaxic frame. A unilateral injection of 6-hydroxydopamine (25 μg/rat) in 5 μl was made at coordinates A 2.4, L 1.7, V 1.9–2.9 above the intra-aural line, which is in the region which includes the zona compacta of the substantia nigra (König & Klippel, 1967).

### *Measurement of turning activity*

Turning activity was measured by direct observation of individual rats placed in an open field measuring 57 cm x 57 cm. Counts were made for 1 min at the times indicated in the figure. Spontaneous locomotor activity was observed in the same open field.

### *Measurement of [$^3$H]-dopamine uptake and release by brain synaptosomes*

*Uptake.* Uptake studies were made on synaptosomes by a method based on that of Snyder & Coyle (1969). The striata from male rats were homogenized with a teflon pestle in a glass grinding vessel in 8 volumes of 0.25M sucrose. Homogenates were centrifuged at 1000 *g* for 10 min at 4°C, and 100 μl of the supernatant synaptosomal fraction were then preincubated with shaking at 37°C for 5 min in an atmosphere of 95% $O_2$ and 5% $CO_2$, in a final volume of 2 ml. The incubation medium was Krebs-Henseleit solution of the following composition (mM) NaCl 117, KCl 5.3, $MgSO_4$ 0.53, $CaCl_2$ 0.95, $NaH_2PO_4$ 0.90, $NaHCO_3$ 25.0, glucose 11.1, ascorbic acid 1.13, disodium edetate 0.13 and pargyline 0.125. After preincubation, [$^3$H]-dopamine was added to make a final concentration in the incubation medium of 0.1 μM. Incubation was continued for 10 min under conditions identical to those of the preincubation. Incubation was stopped by rapid cooling in an ice bath, and the tissue and incubation medium were separated by centrifugation at 50,000 *g* for 30 min at 1–2°C. A sample (0.1 ml) of the supernatant fluid was taken and total radioactivity determined by liquid scintillation counting. The remaining supernatant was discarded and the pellet was rinsed twice with 2 ml ice cold saline. Absolute ethanol (1 ml) was added to the tube containing the pellet, and left to extract at 1–2°C for 16 h; 0.1 ml of the ethanol was taken for liquid scintillation counting. Particle medium ratios were calculated as counts $min^{-1}$ g wet $tissue^{-1}$ divided by counts $min^{-1}$ ml incubation $medium^{-1}$. Blank values from samples identically prepared and treated but incubated at 1–2°C were subtracted and inhibition of uptake was calculated by comparison with samples incubated free of drug. $IC_{50}$ values, defined as the molar concentration of drug producing 50% inhibition of [$^3$H]-dopamine uptake in 10 min were calculated by plotting the % inhibition of uptake against concentration of inhibitor on semilogarithmic paper. At least six determinations were made at each point.

*Release.* The ability of mazindol to release [$^3$H]-dopamine from synaptosomes was studied by a method based on that of Ferris, Tang & Maxwell (1972). Synaptosomes from rat striatum were prepared as described above; 3 ml of the 1000 *g* supernatant fluid containing synaptosomes was incubated with 17 ml incubation medium (composition described above), at 37°C in an atmosphere of 95% $O_2$ and 5% $CO_2$, with shaking, for 10 minutes. [$^3$H]-dopamine (final concentration 0.1 μM) was then added, and incubation continued for 5 minutes. The incubate was then cooled to 1–2°C and centrifuged at 50,000 *g* for 30 min at 1–2°C. The supernatant fluid was discarded, and the pellet was carefully washed twice with 2 ml ice cold saline. The pellet was then resuspended in the original volume (3 ml) of ice cold incubation medium; 100 μl of this preparation was then added to 1.9 ml fresh incubation medium (preincubated for 5 min at 37°C in 95% $O_2$ and 5% $CO_2$) and incubation continued for a further 5 min in either the presence or absence of drug. Incubation was stopped by rapid cooling, and particle : medium ratios were determined as described above. The percentage [$^3$H]-dopamine released in the absence of drug was subtracted from the percentage [$^3$H]-dopamine released in the presence of drug. The corrected percentage of [$^3$H]-dopamine released under the influence of drug alone was then plotted against concentration of drug on semilogarithmic paper, and $RC_{50}$ values (defined as the molar concentration of drug required to release 50% of the [$^3$H]-dopamine from the synaptosomes in 5 min) were determined.

### *Drugs*

The following drugs were used: mazindol (Wander), (+)-amphetamine sulphate, phenoxybenzamine hydrochloride, (SK & F), apomorphine hydrochloride (MacFarlane Smith), pimozide (Janssen), 6-hydroxydopamine dihydrobromide, DL-α-methyl-*p*-tyrosine methyl ester (Sigma), (−)-propranolol (ICI), methergoline (Pharmatalia), reserpine (BDH), pargyline (Abbott), [$^3$H]-dopamine hydrochloride (5.6 Ci/mmol) (The Radiochemical Centre, Amersham).

## Results

### *Antagonism of anorectic responses to mazindol by receptor blockers*

Dose-effect relationships were established for mazindol-induced anorexia in the absence (saline pretreatment) or presence of drugs with receptor blocking activity. From these dose-effect curves $ED_{50}$ values were calculated by the method of Litchfield & Wilcoxon (1949).

Following pretreatment with pimozide, a significant antagonism of mazindol anorexia was seen, and the $ED_{50}$ values were significantly increased. 5-HT receptor blockade, $\alpha$-adrenoceptor block or $\beta$-adrenoceptor block did not antagonize mazindol anorexia (Table 1).

Whereas pimozide and methergoline had no effect, (–)-propranolol (10 mg/kg) and phenoxybenzamine (5 mg/kg) decreased food intake (Table 2). The $ED_{50}$ for mazindol following propranolol was significantly decreased; after phenoxybenzamine pretreatment the $ED_{50}$ value for mazindol was not significantly changed.

While the receptor antagonist experiments indicated that mazindol interacts with dopamine receptors, it was not possible to distinguish from feeding experiments whether the action was by direct receptor stimulation, or indirectly by release and/or block of re-uptake.

### *Animal models used to distinguish between direct and indirect receptor activation*

*Turning in rats with a unilateral substantia nigra lesion.* Mazindol caused a dose-dependent turning towards the lesioned side, and this effect could be antagonized by pretreatment with pimozide (Figure 1). In the same rats, (+)-amphetamine (1–8 mg/kg i.p.) caused a dose-dependent turning towards the lesioned side, while apomorphine (0.1–1.0 mg/kg s.c.) caused a dose-dependent turning away from the lesioned side. In the conventional interpretation of this model (Ungerstedt, 1971) direct receptor stimulant drugs such as apomorphine cause turning away from the lesioned side due to direct stimulation of supersensitive receptors, while indirect receptor stimulant drugs such as amphetamine, cause turning towards the lesioned side by releasing dopamine from the unlesioned side. By analogy in this particular model, it was concluded that mazindol acts indirectly in a manner similar to that of (+)-amphetamine.

**Table 1** Effect of receptor blockers on mazindol-induced anorexia

| *Pretreatment (mg/kg i.p.)* | *Pretreatment time (h)* | *Treatment range of 5 or 6 doses (mg/kg i.p.)* | *$ED_{50}$ (±95% confidence limits) (mg/kp i.p.)* | P |
|---|---|---|---|---|
| Saline | 2 | Mazindol (0.5–8) | 2.4 (5.25–1.10) | |
| Pimozide 0.062 | 2 | Mazindol (1–16) | 5.1 (11.22–2.32) | <0.05 |
| Pimozide 0.125 | 2 | Mazindol (1–16) | 5.8 (12.06–2.79) | <0.05 |
| Pimozide 0.25 | 2 | Mazindol (1–16) | 6.9 (10.42–4.57) | <0.05 |
| Methergoline 0.5 | 2 | Mazindol (0.5–8) | 2.0 (4.58–0.87) | NS |
| Phenoxybenzamine 5 | 1 | Mazindol (0.5–8) | 2.4 (4.12–1.4) | NS |
| Propranolol 10 | 1 | Mazindol (0.5–8) | 0.98 (1.47–0.65) | <0.05 |

$ED_{50}$ values are based on the food eaten during the first hour after presentation of food, 30 min after mazindol treatment.

**Table 2** Effect of receptor blockers on feeding in groups of 6 trained rats

| *Pretreatment (Saline 5 ml/kg or receptor blocker mg/kg i.p.)* | *Pretreatment time (h)* | *Food eaten in the first hour after saline pretreatment (g ± s.e. mean, n = 6)* | *Food eaten in the first hour after receptor blocker pretreatment (g ± s.e. mean, n = 6)* | P |
|---|---|---|---|---|
| Pimozide 0.125 | 2 | 71.5 ± 4.6 | 67.8 ± 3.0 | NS |
| Pimozide 0.25 | 2 | 70.5 ± 2.6 | 75.0 ± 2.5 | NS |
| Methergoline 0.5 | 2 | 60.8 ± 5.8 | 66.0 ± 3.5 | NS |
| Phenoxybenzamine 5 | 1 | 60.7 ± 4.5 | 46.7 ± 4.2 | <0.05 |
| (–)-Propranolol 10 | 1 | 71.4 ± 4.7 | 57.5 ± 4.9 | <0.05 |

Saline, 5 ml/kg was administered 30 min before presentation of food, after the pretreatments as shown.

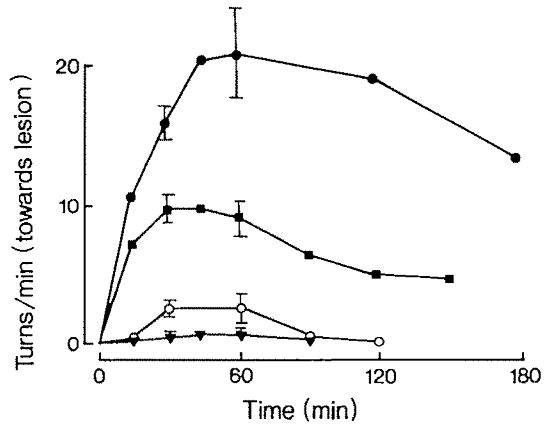


**Figure 1** Effect of mazindol (O) 2 mg/kg, (■) 4 mg/kg, (●) 8 mg/kg i.p., and mazindol (▲) 4 mg/kg i.p. 2 h after pimozide 0.5 mg/kg, on turning behaviour in rats with a unilateral 6-hydroxydopamine lesion in the substantia nigra. $n = 6$. Vertical bars indicate s.e. mean.

*Spontaneous activity in reserpine and α-methyl-p-tyrosine treated animals.* By pretreatment with reserpine (5 mg/kg) 16 h previously and DL-α-methyl-*p*-tyrosine (250 mg/kg) 1 h previously, it is possible to test drugs in animals in which storage and synthesis of catecholamines is severely decreased (Andén, Fuxe, Hökfelt & Rubensson (1967). As shown in Table 3, the response of animals treated with mazindol was indistinguishable from the response observed following (+)-amphetamine. The effect could not be altered by increasing the dose of either mazindol or (+)-amphetamine. By contrast, the direct dopamine receptor stimulant apomorphine caused a dose-dependent increase in motor activity, aggression and chewing activity, some of the animals inflicting major damage to their own hind legs and tails by their compulsive chewing.

The results from this series of experiments indicated that mazindol has indirect dopamine receptor stimulant action, and it was therefore decided to investigate the ability of mazindol to release and block re-uptake of dopamine.

*Effect of mazindol on uptake and release of dopamine in striatal synaptosomes*

Mazindol blocked the uptake of [$^3$H]-dopamine into synaptosomes prepared from rat neostriatum in a concentration-dependent manner (Table 4). At a concentration 50 times greater than that needed to block uptake of dopamine by 50%, mazindol could release 50% of the [$^3$H]-dopamine previously taken up into neostriatal synaptosomes. For comparative purposes, (+)-amphetamine was also tested in this system and it is seen that amphetamine is approximately 3 times more potent as an uptake blocker and 300 times more potent as a releaser of dopamine than mazindol.

## Discussion

Mazindol anorexia was antagonized by a specific dopamine receptor blocking compound, pimozide (Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970). Pimozide also antagonizes (+)-amphetamine-induced anorexia (Kruk, 1973). The antagonism of the

**Table 3** Effect of mazindol, (+)-amphetamine and apomorphine on spontaneous behaviour in rats, pretreated 16 h previously with reserpine (5 mg/kg i.p.) and 1 h previously with DL-α-methyl-*p*-tyrosine (250 mg/kg i.p.)

| *Mazindol (4 mg/kg i.p.)* | *(+)-Amphetamine (4 mg/kg i.p.)* | *Apomorphine (1 mg/kg s.c.)* |
|---|---|---|
| Still, hunched, 'reserpinized' | Still, hunched, 'reserpinized' | Aggressive, vocal, difficult to handle, self-chewing |

Six rats/group.

**Table 4** Effect of mazindol on the uptake and release of [$^3$H]-dopamine by synaptosomes prepared from rat neostriatum

| *Drug* | *$IC_{50}$ uptake* | *$RC_{50}$ release* |
|---|---|---|
| Mazindol | 0.27 ± 0.04 μM (6) | 70 ± 3.5 μM (7) |
| (+)-Amphetamine (base) | 66 ± 15 nM (7) | 0.25 ± 0.22 μM (6) |

anorectic response by pimozide could be overcome by increasing the dose of mazindol, thus showing that the antagonism is at dopamine receptors, is competitive and reversible. This is in agreement with the finding of Barzaghi, Groppetti, Mantegazza & Müller (1973) and Kruk (1973), who found that stimulation of dopamine receptors with a direct receptor agonist, apomorphine, produced anorexia. These authors all concluded that activation of dopamine receptors can mediate anorectic responses.

Activation of 5-HT receptors by direct application of 5-HT (Kruk, 1973) or indirectly by release of 5-HT by drugs such as fenfluramine and norfenfluramine (Clineschmidt, McGuffin & Werner, 1974; Broekkamp, Weemaes & Van Rossum, 1975) can produce anorexia in rats. In the experiments reported here, methergoline, a specific 5-HT receptor blocker (Beretta, Ferrini & Glässer, 1965), did not antagonize the anorectic response to mazindol; thus it was concluded that activation of 5-HT mechanisms is not involved in mediating mazindol anorexia.

Engstrom *et al.* (1975) and Gogerty *et al.* (1975) tentatively suggest that mazindol anorexia may be associated with the ability of mazindol to block noradrenaline uptake, presumably implying that the anorexia is mediated by activation of noradrenaline-sensitive receptors. The lack of antagonism by $\alpha$-adrenoceptor of $\beta$-adrenoceptor blocking agents in the present experiments, argues against noradrenaline having a role in mediating anorectic responses to mazindol. Schmitt (1973) found that neither $\alpha$-adrenoceptor nor $\beta$-adrenoceptor blocking agents antagonized anorectic responses to (+)-amphetamine or fenfluramine, indeed it would be unexpected to find noradrenaline having a role in mediating anorectic responses as it is well documented that direct application of noradrenaline or substances stimulating $\alpha$-adrenoceptors, stimulates feeding in satiated rats, and this effect can be antagonized by $\alpha$-adrenoceptor blocking agents (Booth, 1968; Broekkamp & Van Rossum, 1972; Kruk, 1973).

From the anorexia antagonism studies with specific receptor blockers, it was concluded that mazindol exerts its action through activation of dopamine receptors. Whether this effect is due to a direct or indirect action on these receptors, was initially investigated *in vivo*.

When mazindol was tested in the substantia nigra-lesioned turning rat model (Ungerstedt, 1971) a dose-dependent turning towards the lesioned side was observed. This turning behaviour could be antagonized by pretreatment with pimozide; thus both turning behaviour and anorexia in response to mazindol are mediated by dopamine receptor activation. The direction of turning, namely towards the lesioned side, is the same as is observed with indirectly acting dopamine receptor agonists such as amphetamine which suggests that mazindol has indirect receptor stimulant activity. This conclusion was corroborated by experiments done in rats in which monoamine stores had been depleted with reserpine, and catecholamine synthesis had been inhibited with $\alpha$-methyl-$p$-tyrosine. In this model, the direct dopamine receptor stimulant apomorphine produced exaggerated behavioural stimulation, while mazindol, in common with (+)-amphetamine, was inactive.

The indirect dopamine receptor stimulant activity of mazindol *in vivo*, was confirmed by biochemical studies *in vitro*. Mazindol effectively blocked the uptake of [$^3$H]-dopamine into synaptosomes prepared from rat neostriatum, the inhibition being concentration-dependent. Further, at concentrations higher than those needed to block dopamine uptake *in vitro*, a marked release of [$^3$H]-dopamine from synaptosomes was observed.

Evidence from *in vivo* experiments using mazindol and specific receptor blockers suggests that it has indirect dopamine receptor stimulant properties. *In vitro* experiments show that mazindol causes the release and blocks the uptake of dopamine. From these two lines of evidence it is concluded that the anorectic effect of mazindol is probably mediated by activation of a dopaminergic mechanism.

*Note added after submission of manuscript*

After submission of this manuscript, articles by Carruba, M.O., Groppetti, A., Mantegazza, P., Vicentini, L. & Zambotti, F. (1976). *Br. J. Pharmac.*, **56**, 431–436, and Zambotti, F., Carruba, M.O., Barzaghi, F., Vicentini, L., Groppetti, A. & Mantegazza, P. (1976). *Eur. J. Pharmac.*, **36**, 405–412, have appeared, which agree with our conclusions that behavioural and biochemical actions of mazindol are mediated by dopaminergic mechanisms.

Dr M.R. Zarrindast is a British Council Fellow from the University of Tehran. We thank Dr D. Crowder of Sandoz Products Limited, for gifts of mazindol.

## References

ANDÉN, N.-E., BUTCHER, S.G., CORRODI, H., FUXE, K. & UNGERSTEDT, U. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmac.*, **11**, 303–314.

ANDÉN, N.-E., FUXE, K., HÖKFELT, T. & RUBENSSON, A. (1967). Evidence for dopamine receptor stimulation by apomorphine. *J. Pharm. Pharmac.*, **19**, 627–629.

BARZAGHI, F., GROPPETTI, A., MANTEGAZZA, P. &

MÜLLER, E.E. (1973). Reduction of food intake by apomorphine: a pimozide sensitive effect. *J. Pharm. Pharmac.*, **25**, 909–911.

BERETTA, C., FERRINI, R. & GLÄSSER, A.H. (1965). 1,6-Dimethyl-8 $\beta$-carboxybenzyloxyaminoethyl-10 $\alpha$-ergoline, a potent and long lasting 5-hydroxytryptamine antagonist. *Nature, Lond.*, **207**, 421–422.

BOOTH, D.A. (1968). Mechanism of action of norepinephrine in eliciting an eating response on injection into the rat hypothalamus. *J. Pharmac. exp. Ther.*, **160**, 336–348.

BROEKKAMP, C.L.E. & VAN ROSSUM, J.M. (1972). Clonidine induced intrahypothalamic stimulation of eating in rats. *Psychopharmacologia, Berl.*, **25**, 162–168.

BROEKKAMP, C.L.E., WEEMAES, A.J.M. & VAN ROSSUM, J.M. (1975). The influence of intracerebral injection of noradrenaline, amphetamine, fenfluramine and norfenfluramine on feeding behaviour of rats. In *Recent Advances in Obesity Research*, ed. Howard, A., pp. 380–382. London: Newman.

CLINESCHMIDT, B.V., McGUFFIN, J.C. & WERNER, B.A. (1974). Role of monoamines in the anorexigenic actions of fenfluramine, amphetamine and *p*-chloromethylamphetamine. *Eur. J. Pharmac.*, **27**, 313–323.

ENGSTROM, R.G., KELLY, L.A. & GOGERTY, J.H. (1975). The effects of 5-Hydroxy-5-(4′-Chlorophenyl)-2,3-Dihydro-5H-Imidazo-(2,1-a) Isoindole (Mazindol), on the metabolism of brain norepinephrine. *Arch. int. Pharmacodyn.*, **214**, 308–321.

FERRIS, R.M., TANG, F.L.M. & MAXWELL, R.A. (1972). A comparison of the capacities of isomers of amphetamine, deoxypipradol and methylphenidate to inhibit the uptake of tritiated catecholamines into rat cerebral cortex slices, synaptosomal preparations and rat cerebral cortex, hypothalamus and striatum and into adrenergic nerves of rabbit aorta. *J. Pharmac. exp. Ther.*, **181**, 407–416.

GOGERTY, J.H., PENBERTHY, C., IORIO, L.C. & TRAPOLD, J.H. (1975). Pharmacological analysis of a new anorexic substance: 5-Hydroxy-5-(4′-Chlorophenyl)-2,3-Dihydro-5H-Imidazo-(2,1-a) Isoindole (Mazindol). *Arch. int. Pharmacodyn.*, **214**, 285–307.

KÖNIG, J.F.R. & KLIPPEL, R.A. (1967). *The Rat Brain.* New York: Robert E. Krieger.

KRUK, Z.L. (1973). Dopamine and 5-hydroxytryptamine inhibit feeding in rats. *Nature, New Biol.*, **246**, 52–53.

LITCHFIELD, J.T. & WILCOXON, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmac. exp. Ther.*, **96**, 99–108.

SCHMITT, H. (1973). The influence of agents interfering with catecholamines and 5-hydroxytryptamine on the food suppressant effect of amphetamine and fenfluramine. *J. Pharmac. (Paris)*, **4**, 285–294.

SLANGEN, J.L. & MILLER, N.E. *(1969)*. Pharmacological tests for the function of hypothalamic norepinephrine in eating behaviour. *Physiol. & Behav.*, **4**, 543–552.

SNYDER, S.H. & COYLE, J.T. (1969). Regional differences in $^3$H-norepinephrine and $^3$H-dopamine uptake into rat brain homogenates. *J. Pharmac. exp. Ther.*, **165**, 78–86.

UNGERSTEDT, U. (1971). Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. *Acta physiol. scand.*, **83**, Suppl. 367, 64–94.

*(Received March 24, 1976.*
*Revised May 20, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 373–382

# STRUCTURE-ACTIVITY RELATIONS OF EXCITATORY AMINO ACIDS ON FROG AND RAT SPINAL NEURONES

T.J. BISCOE, R.H. EVANS, P.M. HEADLEY,[1] M.R. MARTIN & J.C. WATKINS
Departments of Physiology and Pharmacology, The Medical School,
University of Bristol, Bristol BS8 1TD

1 A series of compounds structurally related to glutamic acid has been tested on frog and rat spinal neurones. The substances were added to procaine-containing medium bathing the isolated hemisected spinal cord of the frog, and their potencies in depolarizing motoneurones were assessed by the magnitude of the potential produced in the ventral root. The electrophoretic technique was used to administer the substances around single interneurones of the rat spinal cord and the relative potencies of the compounds as excitants assessed by the magnitude of the currents required to produce similar rates of neuronal firing.

2 Parallel structure-activity relations were observed in the two series of experiments, suggesting that the receptors for excitatory amino acids on frog and rat spinal neurones are similar.

3 Quisqualate, domoate and kainate were the strongest excitants in both animals, with potencies around two orders of magnitude higher than that of L-glutamate.

4 2,4,5-Trihydroxyphenylalanine (6-OH-DOPA) was a stronger excitant and L-3,4-dihydroxyphenylalanine (L-DOPA) a weaker excitant than L-glutamate on frog spinal motoneurones. The former compound was also a more potent convulsant than L-glutamate on intraventricular injection into mouse brain. The lack of activity of 6-OH-DOPA on electrophoretic administration was attributed to oxidation.

5 Unlike the majority of amino acid excitants, several of the compounds shown in the present work to have moderate excitatory activity are not anionic at physiological pH. This indicates either that two negatively charged groups are not essential for interaction with a common excitatory receptor, or that more than one type of receptor is involved in the actions demonstrated.

## Introduction

There is mounting evidence that the anions of some acidic amino acids may function as excitatory transmitter substances in the mammalian central nervous system. Although L-glutamate and L-aspartate are the two compounds most generally favoured to perform such a role, structure-activity studies (Curtis, Phillis & Watkins, 1961; Curtis & Watkins, 1963) have revealed several other compounds that must also be considered as possible transmitters (Curtis & Johnston, 1974). A continuing study of structure-activity relations is important not only to extend the list of transmitter candidates, but also to gain insight into the mechanisms underlying the membrane depolarizing effects of these compounds on central neurones.

It was previously shown that the actions of amino acids on neurones of the amphibian isolated hemisected spinal cord, as manifested by changes in the level of ventral root polarization when the substances were added to the perfusion medium (Curtis *et al.*, 1961), were generally consistent with the effects of the substances when applied by micro-electrophoresis around single neurones of the mammalian spinal cord (Curtis & Watkins, 1960, 1963). However, interpretation of the amphibian results was complicated by the fact that the ventral root responses were compounded of both direct actions of the added substances on the motoneurones and indirect effects on neurones forming part of synaptic pathways terminating on motoneurones. This difficulty has recently been overcome by the inclusion in the perfusion medium of procaine, tetrodotoxin or a high $Mg^{2+}$ concentration, all of which prevent synaptically relayed effects, so restricting the

[1] Present address: John Curtin School of Medical Research, The Australian National University, Canberra A.C.T., Australia.

responses of the ventral roots to the direct actions of the amino acids on motoneurones (Barker & Nicoll, 1973; Konishi & Otsuka, 1974; Barker, Nicoll & Padjen, 1975; Evans & Watkins, 1975a). Using the procaine-containing system we have tested an extensive series of excitants covering a wide range of related structures and established an order of potency for the compounds on frog motoneurones. In view of the convenience of the preparation for studying amino acid actions, it was important to investigate if such results might be meaningful in relation to the mammalian central nervous system. Accordingly, a selected series of the same compounds was also tested on rat spinal neurones by the microelectrophoretic technique, and the relative potencies compared with those obtained on the frog. Preliminary reports of this work have been published (Biscoe, Evans, Headley, Martin & Watkins, 1975; Evans & Watkins, 1975b).

## Methods

Amino acids were tested for excitatory activity on the isolated hemisected cord preparation of the frog (Curtis *et al.*, 1961) modified by the inclusion of 1 mM procaine in the perfusion medium (Evans & Watkins, 1975a). The other components of the perfusion medium were (mM): NaCl 111, KCl 2, $CaCl_2$ 2, glucose 12 and tris base 10; the pH was adjusted to 7.5 with 11.3 N HCl. The solution was dripped directly on to the tissue at a rate of 1.5 ml per min, with the temperature maintained at 12.5 ± 0.2°C. Ag/AgCl electrodes were used to record the potentials occurring between the distal end of ventral roots 8 or 9 and the bathing solution; the potential so recorded reflects the level of motoneurone transmembrane polarization. Solutions of the amino acids in the perfusion medium were tested in 1 ml volumes at two concentrations within the range 0.25 μM to 5 mM (pH adjusted to 7.5 with NaOH where necessary) on at least three preparations. The depolarizations produced were matched with those obtained with L-glutamate (0.05–2 mM). Relative potencies were obtained from the ratios of the concentrations producing equal depolarizations. Recovery times, following washout of the substances, were also measured and compared with those observed for L-glutamate.

Many of the compounds were also tested on rat lumbar spinal interneurones by microelectrophoresis (Biscoe, Duggan & Lodge, 1972). Extracellular recordings of the discharge rates of neurones were made with the 4 M NaCl-filled centre barrel of seven barrel micropipettes, and were displayed on a potentiometric pen recorder by conventional techniques. The remaining barrels contained solutions of the mono-sodium salts of the amino acids to be tested; these were prepared by dissolving the free acids in one equivalent of NaOH solution of the desired molarity. Estimates of the excitatory potency of the various amino acids were made by comparing the ejecting currents required to produce approximately equal frequencies of spike discharge. In an attempt to relate the potencies of the strong excitants (such as the anthelmintics kainate, domoate and quisqualate) to that of L-glutamate, comparisons were made on the same cells first of the potencies of the strong excitants with DL-homocysteate (all at 2.5 or 5 mM in 47.5 or 45 mM NaCl, respectively) and then of DL-homocysteate with L-glutamate (both at 200 mM in water). The use of these dilute solutions of the strong excitants was necessary in order to minimize the effects of diffusion from the electrode tips. All other amino acids were used as 200 mM solutions of the mono-sodium salts in water.

In order to gain information about the purity and also about the ionization of the substances, all the amino acids used in this study were subjected to high-voltage paper electrophoresis using buffers of various pH values between 3 and 10 (2000 V/15 min; Whatman No. 1 paper). In the case of 2,4,5-trihydroxyphenylalanine (6-OH-DOPA) and L-3,4-dihydroxyphenylalanine (L-DOPA), 50 mM ascorbic acid was included in the buffers to protect against oxidation. Spots were detected with ninhydrin.

The compounds used, and their sources, were as follows: D- and L-glutamic acid, D- and L-aspartic acid, D-asparagine, L-serine (BDH); kainic acid, L-DOPA, 6-OH-DOPA, D- and L-malic acid, D-asparagine, D-serine (Sigma); DL-homocysteic acid (Calbiochem); quisqualic acid, domoic acid, α-allokainic acid (gifts from Professor T. Takemoto, Tohoku University); DL-2-amino-4-thiosulphonylbutyric acid and L-2-amino-3-thiosulphonylpropionic acid (gifts from Dr C. de Marco, Cagliari); 6-hydroxy-2-pyridylalanine (gift from Dr P.T. Sullivan, Ann Arbor); *O*-sulpho-L-serine (gift from Dr R.A. John, Cardiff); mimosine (gift from Professor K-H. Ling, Taipei); *N*-methyl-D-aspartic acid (prepared by the method of Watkins, 1962).

## Results

### *Anthelmintics*

Quisqualic acid, domoic acid and kainic acid (Table 1, compounds 1, 2 and 3, respectively) are naturally occurring substances which have powerful anthelmintic properties; quisqualic acid occurs in the seeds of the plant genus *Quisqualis* (Takemoto, Takagi, Nakajima & Koiki, 1975; Takemoto, Koike, Nakajima & Arihara, 1975; Takemoto, Nakajima, Arihara & Koiki, 1975), while kainic and domoic acids are found in the marine algae *Digenea simplex* and *Chondria armata* respectively (Ueno, Nawa, Ueganagi, Morimoto, Nakamori & Matsuoka, 1955;

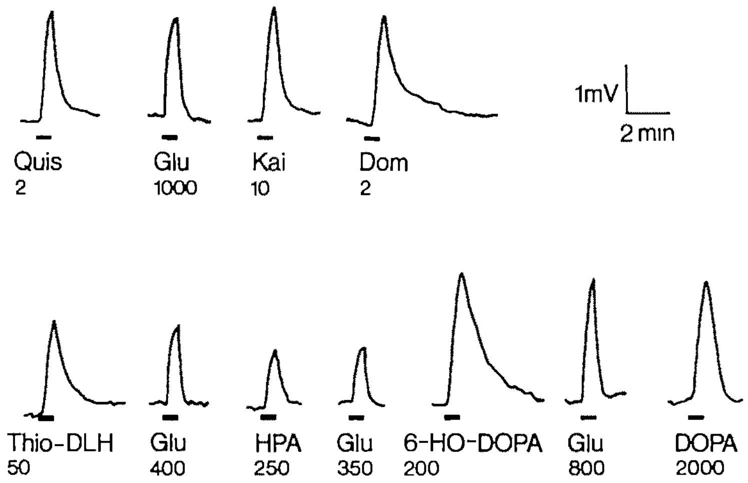


**Figure 1** Ventral root depolarizations produced by excitatory amino acids on the isolated hemisected spinal cord of the frog in the presence of 1 mM procaine. Period of contact of the amino acids with the tissue shown by the horizontal bars. Figures below each compound refer to concentration in μM. Quis, quisqualate; Glu, L-glutamate; Kai, kainate; Dom, domoate; Thio-DLH, DL-2-amino-4-thiosulphonyl-*n*-butyrate; HPA, 1,2-dihydro-2-oxo-6-pyridylalanine; 6-OH-DOPA, 2,4,5-trihydroxyphenylalanine; DOPA, L-3,4-dihydroxyphenylalanine.

Takemoto & Draigo, 1960; Takemoto, Draigo, Kondo & Kondo, 1966). Quisqualate is a potent glutamate-like excitant at the crayfish neuromuscular junction (Shinozaki & Shibuya, 1974) and kainate strongly excites rat cortical (Shinozaki & Konishi, 1970) and cat spinal (Johnston, Curtis, Davies & McCulloch, 1974) neurones. Both compounds are considerably more potent than L-glutamate in these actions.

In the present study, all three amino acids were found to be remarkably potent as excitants of spinal cord neurones in both the frog and rat (Table 1). Concentrations in the range of 0.25–10 μM gave readily measurable depolarizations in the frog ventral root. The actions of these amino acids were more prolonged than that of L-glutamate, persisting for up to 5 min after washout in the case of domoate (Figure 1). The action of α-allo-kainate (Table 1, 13), a stereoisomer of kainate, was considerably weaker than that of kainate in the frog cord, but was similarly prolonged; this compound has also been shown to be less potent than kainate on rat cerebral neurones (Shinozaki & Konishi, 1970).

When tested in the rat spinal cord the three anthelmintics and also α-allo-kainate showed similar relative potencies to those observed in the frog. The excitatory action of domoate and kainate was sometimes prolonged, occasionally lasting for several minutes after termination of the ejection currents. This phenomenon, coupled with the effects of the high retaining currents necessary to prevent diffusional escape of the substances, caused difficulties in the assessment of the potencies of these compounds relative to other amino acid excitants. Two methods were used in an attempt to evaluate relative potencies. With the first method, substances were applied repeatedly for relatively short periods (0.5–1.5 min) until successive applications gave similar responses. This procedure occasionally resulted in maintained low frequency excitation between applications of the longer-acting substances (e.g. kainate and domoate in Figure 2a). Such an effect might be expected to give spuriously high estimates for the relative potencies of these substances; however, equally high or higher potencies relative to DL-homocysteic acid were sometimes observed even in the absence of maintained baseline . excitation (Figure 2b). With the second method (not illustrated), substances were applied continuously over a period of 1–3 min, the ejection currents being adjusted as necessary to achieve a plateau of firing frequency as close as possible to that obtained by similarly prolonged administration of DL-homocysteate. The two methods yielded similar ranges of potency ratios, and results from both are included in the ranges given in Table 1.

### *Sulphur-containing amino acids*

The high activity of DL-2-amino-4-thiosulphonylbutyric acid (De Marco, 1972) (Table 1, 6), observed in both the frog (Figure 1) and rat (Figure 2c) was similar to that of the sulphonic acid analogue DL-homocysteic acid (Table 1, 5). L-2-Amino-3-thiosulphonylpropionic acid (Table 1, 14), which is the L-

**Table 1** Compounds tested on frog and rat spinal neurones

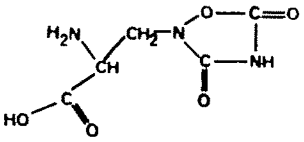
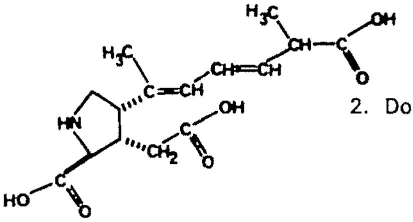
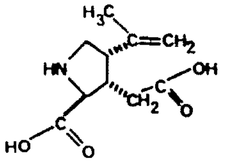
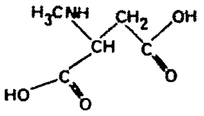
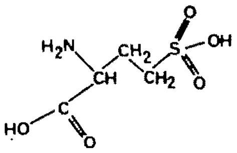
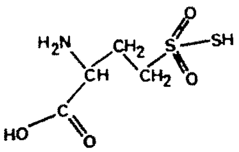
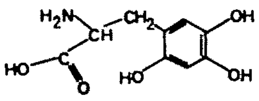
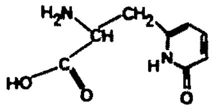
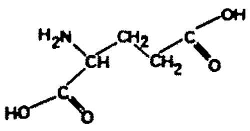
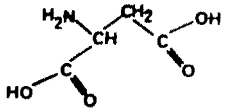
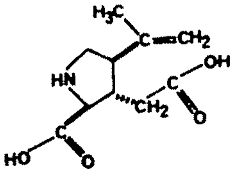
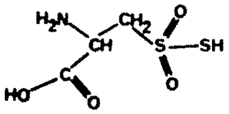
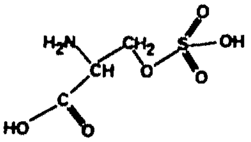
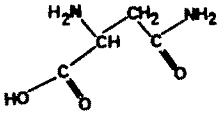
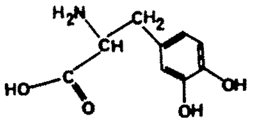
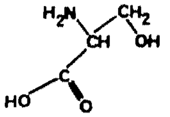
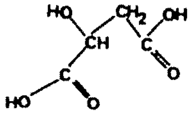
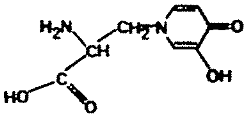
| | | | *Frog* | | *Rat* |
|---|---|---|---|---|---|
| *Structure* | *No.* | *Name* | *Potency** | *Recov.†* | *Potency range** |
|  | 1. | Quisqualic acid | 416±68 | 3.7±0.4 | 22–90 |
|  | 2. | Domoic acid | 280±49 | 8.8±0.8 | 36–190 |
|  | 3. | Kainic acid | 101±18 | 4.7±1.1 | 18–54 |
|  | 4. | *N*-Methyl-D-aspartic acid | 12.4±1.9 | 4.5±0.8 | 4–12 |
|  | 5. | DL-Homocysteic acid | 11.4±1.2 | 3.5±0.8 | 2–5 |
|  | 6. | DL-2-Amino-4-thiosulphonyl butyric acid | 8.3±0.7 | 4.1±0.2 | 4–11 |
|  | 7. | 2,4,5-Trihydroxyphenylalanine (6-OH-DOPA) | 4.3±0.3 | 10.3±2.2 | |
|  | 8. | 1,2-Dihydro-2-oxo-6-pyridylalanine | 0.96±0.15 | 2.3±0.4 | 0.5–2 |
|  | 9. | L-Glutamic acid | 1.0 | 1.0 | 1.0 |
| | 10. | D-Glutamic acid | 2.1±0.6 | 1.3±0.1 | |

**Table 1**—*continued*

| | | | *Frog* | | *Rat* |
|---|---|---|---|---|---|
| *Structure* | *No.* | *Name* | *Potency** | *Recov.†* | *Potency range** |
|  | 11. | L-Aspartic acid | 0.49 ± 0.06 | 1.3 ± 0.2 | |
| | 12. | D-Aspartic acid | 0.75 ± 0.13 | 1.0 ± 0.1 | |
|  | 13. | *α-allo*-Kainic acid | 0.25 ± 0.05 | 6.3 ± 0.8 | 0.5–2 |
|  | 14. | L-2-Amino-3-thiosulphonyl-propionic acid | 0.4 | | 0.1–0.2 |
|  | 15. | *O*-Sulpho-L-serine | 0.46 ± 0.07 | 1.4 ± 0.2 | |
|  | 16. | L-Asparagine | 0.13 ± 0.3 | 2.1 ± 0.1 | |
| | 17. | D-Asparagine | 0.33 ± 0.05 | 2.3 ± 0.2 | |
|  | 18. | L-DOPA | 0.26 ± 0.06 | 2.3 ± 0.2 | 0–0.2 |
|  | 19. | L-Serine | 0.03 ± 0.005 | 2.6 ± 0.6 | |
| | 20. | D-Serine | 0.24 ± 0.02 | 2.4 ± 0.7 | ** |
|  | 21. | L-Malic acid | 0 | | |
| | 22. | D-Malic acid | 0.14 ± 0.03 | 2.8 ± 0.2 | |
|  | 23. | Mimosine | 0.09 ± 0.01 | 3.9 ± 0.5 | |

* Relative to L-Glutamate = 1.0. † Time from peak response to 90% of full recovery expressed as a ratio to the recovery from L-glutamate-induced depolarizations of equal magnitude. ** Paroxysmal excitation, presumably due to co-administered $H^+$ ions.

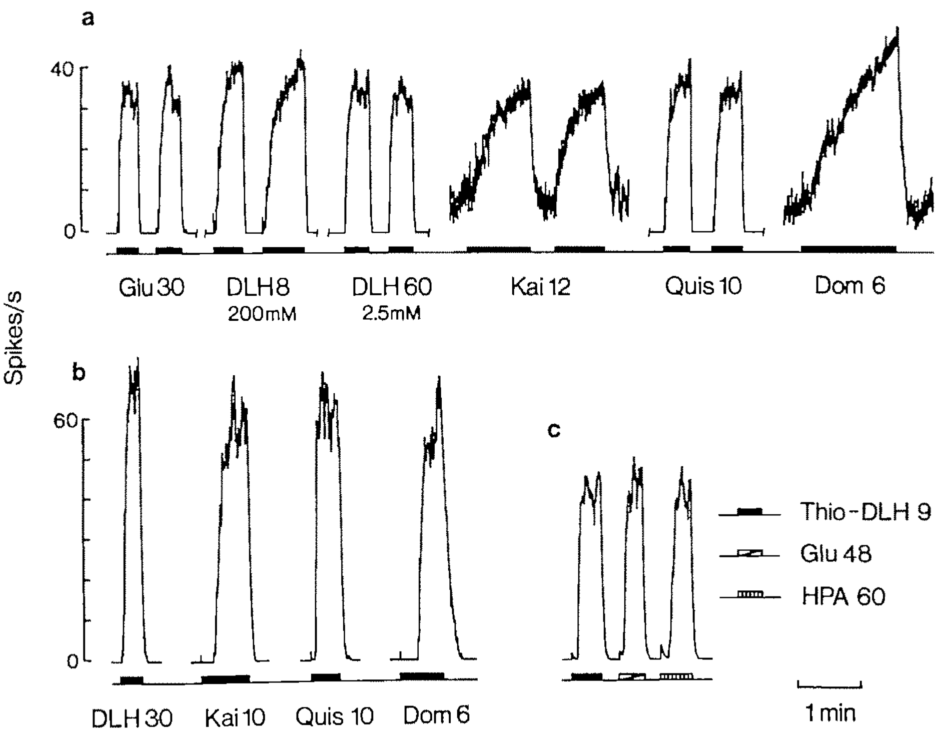


**Figure 2** Chart recordings of the excitation of three different rat spinal interneurones by electrophoretically administered amino acids. Ordinates, spike frequency; abscissae, time (min). Horizontal bars show duration of administration. The compounds and the electrophoretic current (nA) are indicated below each bar. (a) Showing method of matching responses of the anthelmintics, kainate, quisqualate, and domoate (all 2.5 mM in 147.5 mM NaCl) with DL-homocysteate (DLH, 2.5 mM in 147.5 mM NaCl and 200 mM in $H_2O$) and with L-glutamate (200 mM in $H_2O$). Note continued base line excitation produced by kainate and domoate. (b) Similar to (a), but no base line excitation produced in this case. (DLH solution, 200 mM in $H_2O$, anthelmintic solutions as in (a).) (c) Matched responses for two other amino acids and L-glutamate (all 200 mM in $H_2O$). Abbreviations as in Figure 1.

form of the lower homologue of compound 6, and the thio-acid analogue of the moderately strong excitant L-cysteic acid (Curtis & Watkins, 1960; 1963; Curtis, Phillis & Watkins, 1960; 1961) was a much weaker excitant than compound 6 in both species; it was not compared with L-cysteic acid. *O*-Sulpho-L-serine (Table 1, 15), an irreversible inhibitor of aspartate aminotransferase (John & Fasella, 1969), was also a weak excitant in the frog cord, corresponding to the weak action of this compound on cat spinal neurones (Curtis, Duggan, Felix, Johnston, Tebēcis & Watkins, 1972); it was not tested on rat neurones.

### *Aromatic amino acids*

A number of polyhydroxyphenyl and hydroxypyridyl amino acids showed a range of excitatory activity. 6-OH-DOPA (Table 1, 7) was the strongest excitant of this type when tested on the frog cord where it had a very prolonged action (Figure 1). No activity could be demonstrated on rat spinal neurones when the substance was tested electrophoretically. This lack of effect in the rat was probably due to oxidation of the compound prior to or during its electrophoretic administration from alkaline solutions (pH 8–9) of the monosodium salt, since stock solutions of similar pH were shown to lose activity at the rate of about 4% per min when tested on the frog spinal cord. This assumption was supported by the effects of fresh solutions of 6-OH-DOPA which were intraventricularly injected into mice by the method of Crawford (1963). The compound (10 µmol/ml) was dissolved in 150 mM NaCl containing 10 mM NaOH, the solution was adjusted to pH 7.4 with HCl, and 10- or 20-µl volumes were injected immediately. Convulsions occurred within a few seconds. When tested concurrently by the same method, DL-homocysteate was more potent and L-glutamate less potent than 6-OH-DOPA.

Three other compounds in this group showed activity both in the superfusion experiments on the frog spinal cord and by the electrophoretic method in the rat spinal cord. L-DOPA (Table 1, 18; Figure 1), which has been shown to be a weak excitant of mammalian central neurones (Krnjević & Phillis, 1963) had weak activity in both species in the present studies. Two pyridine-related compounds, 1,2-dihydro-2-oxo-6-pyridylalanine (Table 1, 8; Figure 1 and 2c), which is the stable (keto) form of 6-hydroxy-2-pyridylalanine, and mimosine (Table 1, 23), were also excitants. The former compound, a synthetic substance (Sullivan, Sullivan & Norton, 1971), was approximately equi-effective with L-glutamate on both frog and rat spinal neurones. In the rat, the substance gave similar results when administered electrophoretically either with anionic currents from solutions of the sodium salt at pH 9 or with cationic currents from solutions of the amino acid adjusted to pH 3 with HCl. Mimosine, a toxic substance which occurs in leguminous plants (De Wreede & Wayman, 1970) was not tested in the rat, but has been reported to be an excitant of cat spinal neurones (Curtis *et al.*, 1972). It was considerably weaker than L-glutamate in depolarizing frog motoneurones.

### *Other compounds*

(a) *N*-Methyl-D-aspartate. This compound (Table 1; 4) was more potent and had a longer lasting effect than L-glutamate on both frog and rat neurones, in accordance with previous studies (Curtis *et al.*, 1961; Curtis & Watkins, 1963; Crawford & Curtis, 1964). However, its potency relative to L-glutamate was considerably lower than that observed in non-procaine treated (unblocked) amphibian spinal cord preparations (Curtis *et al.*, 1961).

(b) D-Glutamate, L- and D-aspartate. These compounds (Table 1; 10, 11 and 12) were tested only on frog spinal neurones in the present study; their relative potencies were within the ranges of those observed in other investigations in both mammals (Curtis & Watkins, 1960; 1963; Crawford & Curtis, 1964) and amphibia (Curtis *et al.*, 1961) though only in the latter study was the mean potency of D-glutamate higher than that of the L-isomer as also found in the present work.

(c) L- and D-Asparagine. The L form (Table 1; 16) had a very weak depolarizing action on the frog cord but the D form (Table 1; 17) was somewhat stronger, being about one third as potent as L-glutamate. This finding conforms with those of previous studies on unblocked amphibian spinal cord (Curtis *et al.*, 1961). Excitatory actions have also been observed in electrophoretic studies in the cat (Krnjević & Phillis, 1963; Crawford & Curtis, 1964) when the substances were administered as cations from acidic solutions, but the actions in those cases were dissimilar to that of L-glutamate and may have involved wholly or in part the effects of raised extracellular pH. Hydrogen ions are co-administered from acidic solutions by electrophoresis, and are also liberated from the cationic form of neutral amino acids when these enter the extracellular fluid (Curtis & Watkins, 1960). The two forms of asparagine were not tested in the present electrophoretic studies on the rat spinal cord.

(d) L- and D-Serine (Table 1, 19 & 20). In the present experiments on the frog cord the L form had only a very weak depolarizing action while the D form was more potent, being about one quarter as strong as L-glutamate. This finding is in agreement with previous studies on the unblocked amphibian spinal cord (Curtis *et al.*, 1961). When passed as a cation from solutions of pH 3 in electrophoretic experiments on the rat, D-serine caused delayed high-frequency discharges which could not be clearly differentiated from $H^+$ ion effects. L-Serine was not tested on rat spinal neurones, but has previously been found to have a strychnine-sensitive depressant action in the cat spinal cord (Curtis, Hösli & Johnston, 1968).

(e) L- and D-Malic acid (Table 1, 21 & 22). On the frog spinal cord, the D form had a weak depolarizing action, but no activity was demonstrated with the L form (5 mM). Neither compound was tested on rat spinal neurones. These compounds have previously been found to be weakly excitatory in the unblocked amphibian spinal cord, the D form again being the more potent (Curtis *et al.*, 1961), but neither compound had any detectable action when administered electrophoretically around cat spinal neurones (Curtis & Watkins, 1960).

## Discussion

A close parallelism has been observed between the relative potencies of a range of amino acid excitants tested on frog and rat spinal neurones. The only major difference observed in the two series of experiments was in the ineffectiveness of 6-OH-DOPA on micro-electrophoretic administration around rat spinal neurones. However, this anomaly was readily explained by the instability of the compound, as discussed in the Results section. It can thus be concluded that the receptors for excitatory amino acids on spinal neurones of the two species are similar.

From the structure-activity relations summarized in Table 1, it appears that a variety of terminal groups can replace the ω-carboxylic terminal of glutamate or aspartate with retention, or indeed in many cases pronounced enhancement of excitatory activity. The

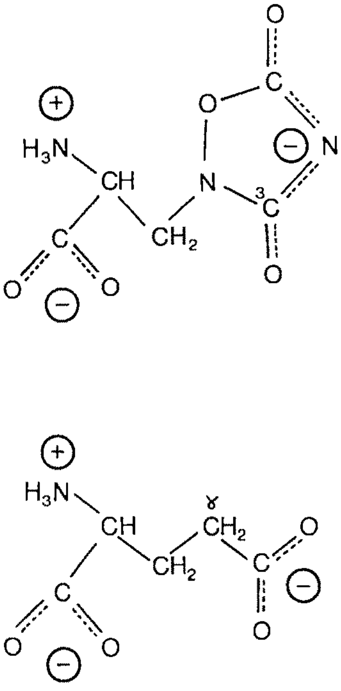


**Figure 3** Proposed anionic structure of quisqualate (above) in comparison with glutamate (below).

allowable variation embraces carboxylic, sulphonic, thiosulphonic, phenolic, hydroxypyridine (keto-form) and the unusual heterocyclic (oxadiazolidine) dione system of quisqualic acid. Other examples have been quoted in our preliminary communication (Biscoe *et al.*, 1975).

Quisqualate, domoate and kainate were by far the most potent excitants. Experiments with high-voltage paper electrophoresis showed that all three substances are anionic at physiological pH. In the case of quisqualic acid (Table 1; 1) this observation presumably indicates that the ring system loses the sole proton and becomes negatively charged, the ionized form probably being stabilized by a resonance hybrid structure involving the carbonyl groups. Thus the charge may be distributed between the nitrogen atom and the two oxygen atoms (Figure 3). Of these three atoms, the C$_3$-oxygen atom seems particularly favourably situated with respect to the amino and carboxylate groups, since in certain conformations of the molecule, this oxygen atom can be isosteric with one of the γ-carboxylate oxygen atoms of glutamate (Figure 3).

The high activity of domoate and kainate (Table 1; 2 and 3 respectively) taken in conjunction with the low activity of α-allo-kainate (Table 1; 13) and also of dihydrokainate (Johnston *et al.*, 1974), suggests that the potency of the former two substances is associated with unsaturation in the side chain and a *cis*-relation between the C$_3$ and C$_4$ substituents. The *cis* relationship of these substituents results in a mutual steric hindrance which greatly restricts the possible conformations of both substituents. It is therefore possible that in the relatively fixed conformation of the C$_3$ side chain, the carboxyl group is particularly well oriented with respect to a corresponding electropositive region of the receptor. Alternatively, or in addition, the unsaturated groups may themselves bind to lipophilic areas close to the electropositive (and presumably hydrophilic) regions of the receptor, so stabilizing the complex.

Among the other compounds tested, most interest attaches to the activity of certain pyridol-related compounds (Table 1; 8 and 23) and particularly of the phenolic substances, 6-OH-DOPA and L-DOPA (Table 1; 7 and 18, respectively). Our findings in regard to the latter compound confirm and extend the observations of Krnjević & Phillis (1963) on neurones in the cat cerebral cortex. Whether this direct excitatory effect of L-DOPA on central neurones is relevant to the therapeutic action of the substance in the treatment of Parkinsonism is a question warranting further investigation.

An important aspect of the activity of the phenolic and pyridol-related compounds is that, on the basis of paper electrophoresis experiments, only mimosine (Table 1; 23), which is a very weak excitant, carries a net negative (anionic) charge at physiological pH, and the ionization of the ring-attached oxygen atoms is only partial even in this case. It has previously been considered (Curtis & Watkins, 1960) that glutamate-like activity requires one cationic and two anionic groups in the active form of the molecule. These groups were envisaged to interact with oppositely charged groups in the receptor molecule. It seems possible, therefore, that if these aromatic compounds do indeed interact with L-glutamate (or L-aspartate) receptors, they do so *via* a metal ion component of the receptor (e.g. Ca$^{2+}$) which could conceivably form coordinate bonds with undissociated hydroxyl groups (with displacement of a proton) as well as with ionized oxygen functions. The actions of asparagine and serine (mainly the D form in both cases) might be explained on a similar basis; in this case the coordinate link with the receptor metal ion would involve the ω-carboxamide and alcoholic OH groups, respectively. The suggestion that bound Ca$^{2+}$ may be an integral part of excitatory amino acid receptors has previously been made (Curtis & Watkins, 1960; Curtis *et al.*, 1961; Watkins, 1965).

The duration of action of these compounds on the frog spinal cord (Table 1) appeared to be independent of depolarizing potency. In both the frog and rat experiments, slower recoveries were generally observed in the case of the more lipophilic compounds. Similarly, it was previously noted that *N*-

alkyl derivatives of D-aspartic acid had durations of action which were directly related to the length of the alkyl chain (Curtis & Watkins, 1963).

In spite of the similarity of the chemical structure of the compounds and of the nature of the observed responses, it should be emphasized that the present experiments do not provide any evidence as to whether the substances all act on the same receptors, or whether they induce the same ionic permeability changes in the neuronal membrane. These considerations must await the development of specific antagonists of amino acid actions, and/or a detailed study of the ionic basis of the potential changes produced in each case.

The work was supported by grants from the Medical Research Council and the Wellcome Trust. P.M.H. was a Wellcome Trust Research Fellow. Skilled technical assistance was provided by Miss E.M. Thornton and Mr D.J. Oakes.

## References

BARKER, J.L. & NICOLL, R.A. (1973). The pharmacology and ionic dependency of amino acid responses in the frog spinal cord. *J. Physiol., Lond.*, **228**, 259–277.

BARKER, J.L., NICOLL, R.A. & PADJEN, A. (1975). Studies on convulsants in the isolated frog spinal cord. II. Effects on root potentials. *J. Physiol., Lond.*, **245**, 537–548.

BISCOE, T.J., DUGGAN, A. W. & LODGE, D. (1972). Antagonism between bicuculline, strychnine and picrotoxin and depressant amino acids in the rat nervous system. *Comp. gen. Pharmac.*, **3**, 423–433.

BISCOE, T.J., EVANS, R.H., HEADLEY, P.M., MARTIN, M.R. & WATKINS, J.C. (1975). Domoic and quisqualic acids as potent amino acid excitants of frog and rat spinal neurones. *Nature, Lond.*, **255**, 166–167.

CRAWFORD, J.M. (1963). The effect upon mice of intraventricular injection of excitant and depressant amino acids. *Biochem. Pharmac.*, **12**, 1443–1444.

CRAWFORD, J.M. & CURTIS, D.R. (1964). The excitation and depression of mammalian cortical neurones by amino acids. *Br. J. Pharmac. Chemother.*, **23**, 313–329.

CURTIS, D.R., DUGGAN, A.W., FELIX, D., JOHNSTON, G.A.R., TEBĒCIS, A.K. & WATKINS, J.C. (1972). Excitation of mammalian central neurones by acidic amino acids. *Brain Res.*, **41**, 283–301.

CURTIS, D.R., HÖSLI, L. & JOHNSTON, G.A.R. (1968). A pharmacological study of the depression of spinal neurones by glycine and related amino acids. *Exp. Brain Res.*, **6**, 1–18.

CURTIS, D.R. & JOHNSTON, G.A.R. (1974). Amino acid transmitters in the mammalian central nervous system. *Ergebn. Physiol.*, **69**, 97–188.

CURTIS, D.R., PHILLIS, J.W. & WATKINS, J.C. (1960). The chemical excitation of spinal neurones by certain acidic amino acids. *J. Physiol., Lond.*, **150**, 656–682.

CURTIS, D.R., PHILLIS, J.W. & WATKINS, J.C. (1961). Actions of amino acids on the isolated hemisected spinal cord of the toad. *Br. J. Pharmac. Chemother.*, **16**, 262–281.

CURTIS, D.R. & WATKINS, J.C. (1960). Excitation and depression of spinal neurones by structurally related amino acids. *J. Neurochem.*, **6**, 117–141.

CURTIS, D.R. & WATKINS, J.C. (1963). Acidic amino acids with strong excitatory actions on mammalian neurones. *J. Physiol., Lond.*, **166**, 1–14.

DE MARCO, C. (1972). Preparation and chromatographic properties of 2-amino-4-thiosulphonylbutyric acid. *Analyt. Biochem.*, **48**, 346–352.

DE WREEDE, S. & WAYMAN, O. (1970). Effect of mimosine on the rat foetus. *Teratology*, **3**, 21–28.

EVANS, R.H. & WATKINS, J.C. (1975a). Ventral root responses of the hemisected amphibian spinal cord to perfused amino acids in the presence of procaine. *Br. J. Pharmac.*, **55**, 519–526.

EVANS, R.H. & WATKINS, J.C. (1975b). Amino acid receptors on frog spinal motoneurones. *Br. J. Pharmac.*, **54**, 238P.

JOHN, R.A. & FASELLA, P. (1969). The reaction of L-serine *O*-sulphate with aspartate aminotransferase. *Biochemistry*, **8**, 4477–4482.

JOHNSTON, G.A.R., CURTIS, D.R., DAVIES, J. & McCULLOCH, R.M. (1974). Spinal interneurone excitation by conformationally restricted analogues of L-glutamic acid. *Nature, Lond.*, **248**, 804–805.

KONISHI, S. & OTSUKA, M. (1974). The effects of substance P and other peptides on spinal neurons of the frog. *Brain Res.*, **65**, 397–410.

KRNJEVIĆ, K. & PHILLIS, J.W. (1963). Actions of certain amines on cerebral cortical neurones. *Br. J. Pharmac. Chemother.*, **20**, 471–490.

SHINOZAKI, H. & KONISHI, S. (1970). Actions of several anthelmintics and insecticides on rat cortical neurones. *Brain Res.*, **24**, 368–371.

SHINOZAKI, H. & SHIBUYA, I. (1974). A new potent excitant, quisqualic acid; effects on crayfish neuromuscular junction. *Neuropharmac.*, **13**, 665–672.

SULLIVAN, P.T., SULLIVAN, C.B. & NORTON, S.J. (1971). α-Fluoro and α-hydroxypyridylalanines. *J. med. Chem.*, **14**, 211–214.

TAKEMOTO, T. & DRAIGO, K. (1960). Constituents of *Chondria armata* and their pharmacological effects. *Arch. Pharm.*, **293**, 627–633.

TAKEMOTO, T., DRAIGO, K., KONDO, Y. & KONDO, K. (1966). Constituents of *Chondria armata*. VIII. On the structure of domoic acid. *Yakugaku Zasshi*, **86**, 874–877.

TAKEMOTO, T., KOIKE, K., NAKAJIMA, T. & ARIHARA, S. (1975). Studies on the constituents of *Quisqualis fructus*. III. Synthesis of quisqualic acid and the related compounds. *Yakugaku Zasshi*, **95**, 448–452.

TAKEMOTO, T., NAKAJIMA, T., ARIHARA, S. & KOIKI, K.

(1975). Studies on the constituents of *Quisqualis fructus*. II. Structure of quisqualic acid. *Yakugaku Zasshi*, **95**, 326–332.

TAKEMOTO, T., TAKAGI, N., NAKAJIMA, T. & KOIKI, K. (1975). Studies on the constituents of *Quisqualis fructus*. I. On the amino acids. *Yakugaku Zasshi*, **95**, 176–179.

UENO, Y., NAWA, H., UEGANAGI, J., MORIMOTO, H., NAKAMORI, R. & MATSUOKA, T. (1955). Studies on the active components of *Digenea simplex:* Ag and related compounds. *J. Pharmac. Soc. Japan*, **75**, 807–844.

WATKINS, J.C. (1962). The synthesis of some acidic amino acids possessing neuropharmacological activity. *J. med. (pharm.) Chem.*, **5**, 1187–1199.

WATKINS, J.C. (1965). Pharmacological receptors and general permeability phenomena of cell membranes. *J. theoret. Biol.*, **9**, 37–50.

*(Received March 26, 1976.*
*Revised May 5, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 383–393

# ANALGESIA PRODUCED BY MORPHINE WHEN ACTING FROM THE LIQUOR SPACE

P.K. DEY & W. FELDBERG
National Institute for Medical Research, Mill Hill, London NW7 1AA

1 In cats analgesia was produced by morphine sulphate introduced into different parts of the liquor space in doses too small to be effective on intravenous injection. Analgesia was measured with the tail pinch method of Russell & Tate (1975).

2 On infusion into the fourth ventricle or into the subarachnoid space beneath the ventral surface of the brain stem caudal to the pons, doses of 100 to 200 μg of morphine sulphate were sufficient to produce strong long-lasting analgesia. On injection into the cisterna magna somewhat larger doses (400 to 800 μg) were required.

3 It is concluded that the site where morphine acts when producing analgesia in all three circumstances is at the ventral surface of the brain stem.

4 The possibility is discussed that the structures acted upon are tryptaminergic nerve fibres. They arise from the raphe nuclei, belong to a descending inhibitory pathway, and on their way to the spinal cord, reach the ventral surface of the brain stem lateral to each pyramid, where they could be reached and acted upon by the morphine. This theory postulates a morphine sensitivity of tryptaminergic nerve fibres.

## Introduction

There are probably several sites at which morphine can act when producing analgesia, but one site appears to be the ventral surface of the brain stem. This conclusion is the outcome of the present experiments on cats in which the analgesic effect of morphine was examined on its introduction into different parts of the liquor space.

Recently, the hyperglycaemia produced by morphine injected into the cerebral ventricles of cats was found to result from an action on structures at the ventral surface of the brain stem, and the possibility was discussed that the same site, though not necessarily the same structures may be responsible for the analgesia that morphine produces on intraventricular injection (Feldberg & Gupta, 1974; Dey & Feldberg, 1975a; Dey, Feldberg & Wendlandt, 1975). There are in fact two observations on the analgesic action itself which could be readily explained in this way, those of Herz, Albus, Metys, Schubert & Teschemacher (1970) and those of Satoh & Takagi (1971). When viewed in conjunction, these two observations suggest the ventral surface of the brain stem as a site of the analgesic action, and perhaps not only when the morphine is injected into the cerebral ventricles, but also when injected intravenously.

Herz *et al.* (1970) showed that small doses of morphine injected into the aqueduct or fourth ventricle of rabbits inhibited the nociceptive response to electrical stimulation of the tooth pulp. They concluded that the analgesic effect was due to an action on the periaqueductal grey matter and on structures in the floor of the fourth ventricle. An action on the ventral surface of the brain stem was not envisaged although this site is readily reached by these injections, since the morphine passes into the subarachnoid space solely through the lateral recesses at the sides of the fourth ventricle and then at once reaches the ventral surface. Rabbits have no foramen of Magendie through which the cisterna magna could be reached. This foramen exists in primates only.

Satoh & Takagi (1971) stimulated the splanchnic nerves in cats and recorded the afferent volleys from the ventrolateral funiculi higher up in the spinal cord. These funiculi are thought to conduct visceral pain. The volleys were suppressed by intravenous injection of 4 mg of morphine and remained suppressed when the brain stem was transected at a level just caudal to the trapezoid bodies. But they were restored when the transection was made about 4 mm more caudally. The inhibitory effect of morphine on the afferent pain impulses must therefore have taken place within a 4 mm long block of brain stem, at least with the dose of morphine used, because in larger doses morphine acted on the spinal cord as well. This 4 mm long block did not include the aqueduct or the anterior half of the fourth ventricle which in the experiments of Herz *et al.* (1970) would have been reached by the injections into the aqueduct, but it included the posterior half.

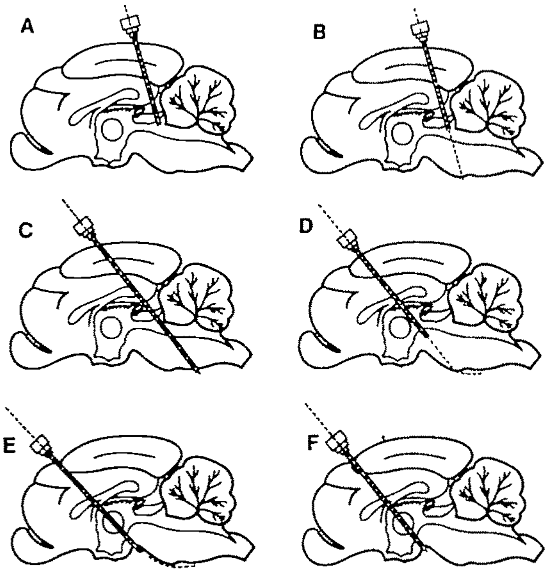


**Figure 1** Diagrams of midsagittal sections of cat's brain with implanted Collison cannulae to illustrate the methods used for infusion of morphine into the fourth ventricle (A) or into the subarachnoid space beneath the ventral surface of the brain stem (B to F). The interrupted lines indicate the hollow needle inserted into and beyond the cannula shaft for the microinfusion of morphine. (For details see text.)

However, this part of the fourth ventricle is not usually reached by intraventricular injections since the lateral recesses through which the fluid passes into the subarachnoid space are situated in the middle of the ventricle. If morphine were to act at the same site in both series of experiments, the site would have to be on the ventral surface in a region caudal to the trapezoid bodies, a possibility not envisaged by either group of workers. This site is readily reached by intraventricular injections and forms the ventral surface of the 4 mm long block in the experiments of Satoh & Takagi (1971). It is also the site at which morphine apparently acts in the present experiments.

Some of the results have been presented to the Physiological Society (Dey & Feldberg, 1975b).

## Methods

The experiments were done on cats of either sex weighing between 3 and 3.5 kg. For infusion of morphine into the fourth ventricle or into the subarachnoid space beneath the ventral surface of the brain stem either caudal to the pons or into the fossa interpeduncularis, a Collison cannula with a stilette inserted into its shaft was implanted in an aseptic operation under pentobarbitone sodium anaesthesia. After recovery from the operation the morphine was infused through these cannulae in a volume of 40 µl by a microinfusion pump which delivered this volume from a 100 µl syringe in 4 min 20 seconds. For the microinfusions the stilette was removed and a hollow stainless-steel needle (28 gauge) was inserted instead. The needle was connected to the syringe by a length of Polythene tubing, needle and tubing being filled with the solution to be infused. The general procedures of the operations as well as the infusions were those described previously (Dey *et al.*, 1975); details varied with the different implantations.

### *Infusion into the fourth ventricle*

The Collison cannula was implanted in such a way that its tip lay in the ventricle near the end of the aqueduct. The method of implantation was the same as described by Feldberg & Gupta (1974) except that the cannula, which had a shaft 22 mm long was inserted at an angle of 28° instead of 20° directed posteriorly, insertion was in the mid line 7 mm anterior to the interaural line. The position of the cannular is shown in diagram A of Figure 1. The hollow stainless-steel needle was inserted through the entire length of the shaft and 1 mm beyond it.

### *Infusion into the subarachnoid space caudal to the pons*

Four methods were used which are illustrated by diagrams B to E of Figure 1. Diagram B shows the method in which the Collison cannula was implanted into the fourth ventricle, but for the infusion the hollow stainless steel needle was inserted not only throughout the entire length of the shaft but also through the brain stem until the tip hit the bone. It was then withdrawn 1 mm before infusion began. The hollow needle was inserted under Althesin (2 mg/kg i.v.) anaesthesia lasting not longer than a few minutes. This short anaesthesia did not interfere with the subsequent testing for analgesia after infusion. Diagram C illustrates the method previously described by Dey *et al.* (1975) in which the Collison cannula with a shaft 37 mm long was implanted 1 mm lateral to the mid line and 13 mm anterior to the interaural line at an angle of 33° directed posteriorly so that the tip ended just beneath the ventral surface of the brain stem. Diagrams D and E illustrate two methods in which the hollow needle inserted into the cannula was allowed to glide 0.5 to 1 cm along the dura under the brain stem so that the tip lay in or a little beyond the region of the trapezoid bodies. The Collison cannulae with a shaft 28 mm long were implanted in the mid line in method D, 20 mm, and in method E, 23 mm, anterior to the interaural line at an angle of 40° directed posteriorly. In method D the tip of the cannula ended within the brain stem and the hollow needle usually bent when, on being lowered (during short-lasting Althesin anaesthesia) it reached the bone; it then glided along the dura. This gliding was facilitated in method E by preparing the tip of the cannula so that on insertion, the part which would be nearest to the bone was deflected slightly upwards and extended 1 mm beyond the part facing the brain stem. Before the Collison cannula was fixed to the skull with acrylic cement, the gliding of the inserted hollow needle along the dura was tested because in method E, the tip of the cannula, or of the needle, sometimes hit the bone just on the sella turcica and then the needle did not glide along the dura. When this happened the cannula was withdrawn and reinserted 1 mm more caudally.

### *Infusion into the fossa interpeduncularis*

The method described by Dey *et al.* (1975) was used. The shaft of the Collison cannula was 27 mm long and the position of the cannula is shown in diagram F of Figure 1.

At the end of the experiments, 40 µl of 0.8% bromophenol blue solution was infused through the Collison cannulae following the same procedure as for the morphine infusions. Fifteen minutes later, the cats were anaesthetized with intraperitoneal pentobarbitone sodium and after another few minutes the head was perfused from the aorta with 13% formalin solution. The brain was removed and with the naked eye the staining was observed of the dura, the walls of the ventricles and the surface of the brain stem. On infusion into the fourth ventricle there was no staining of the aqueduct or third ventricle, but deep staining of the anterior half of the floor of the fourth ventricle which contrasted sharply with the unstained posterior half. The lateral recesses and the dura beneath the brain stem were well stained; there was faint staining of its ventral surface; the basilar artery and its branches had usually taken up the dye and were lightly stained. With methods B, D and E, the dura beneath the brain stem was deeply stained, the staining extending from the sella turcica to the region of the medulla and sometimes of the cervical cord. The ventral surface of the brain stem and the basilar artery with its branches were faintly stained. There was no staining along the cannula tract in the brain stem or in the floor of the fourth ventricle. With method C the staining was either the same as described for methods B, D and E or, more usually, the dye had not freely entered the subarachnoid space and had deeply stained a region of varying size on the ventral surface around the tip of the cannula. In addition, the tract of the cannula either at its lower end or throughout its entire length was deeply stained, and sometimes the dye had even passed into the fourth ventricle and faintly stained the anterior half of its floor. There was no difference in the results obtained with morphine whether the tract and the floor of the fourth ventricle had become stained or not. Nevertheless the experiments in which the floor of the fourth ventricle became stained were not included in Table 1 which summarizes the results obtained on infusion of morphine into the subarachnoid space caudal to the pons. But the table includes two experiments (Nos. 9 and 12) in which the whole needle tract was found to be stained.

### *Injection into the cisterna magna*

A Collison cannula was fixed to the back of the skull in an aseptic operation under pentobarbitone sodium anaesthesia so that the tip of the cannula shaft rested just above the atlanto-occipital membrane. For the injection, a hollow needle was lowered through the shaft piercing the membrane. The method has been described in detail elsewhere (Feldberg, Gupta, Milton & Wendlandt, 1973) and the position of the cannula is shown in the lower diagram of Figure 4. The morphine was injected in a volume of 0.1 ml and washed in by another 0.1 ml 0.9% w/v NaCl solution (saline). With this method of injection no morphine enters the cerebral ventricles which remain unstained when 0.8% bromophenol blue instead of morphine is similarly injected.

### *Testing for analgesia*

The testing was carried out with the cat placed in a 50 x 50 cm and 53 cm high wire cage, the whole front of which could be opened. The tail pinch method of Russell & Tate (1975) was used in which a small diameter rod presses across the root of the tail. The method allows the application of graded pressure for a short time; the device works hydraulically and the hydraulic pressure is measured by a Bourdon gauge in kg/cm$^2$. Although this pressure is not identical with the force exerted on the tail it is proportional to it and can therefore be used, without conversion, for measurements of analgesia. In the present experiments pressure was maintained for 5 s, and with the special instrument used no response was produced by a pressure which varied in different experiments between 0.2 and 0.8 kg/cm$^2$ (mean 0.4 kg/cm$^2$). When a response was obtained by applying stronger pressure it was classified as either weak, medium or strong.

A weak response which need not always be one to pain, consisted of slight tension in the animal, usually in the hindquarters, or of lifting of the hindquarters, or of the cat slowly getting up and slowly moving about, or of turning the head toward the pincher, staring at it, but not trying to bite it, or of holding the pincher with a forepaw without attempting to remove it. Sometimes the cat got up quickly for a moment at the beginning of the pressure, but then relaxed; this, too, was classified as a weak response.

A strong response suggested intense pain. It consisted of the cat jumping up, occasionally somer-

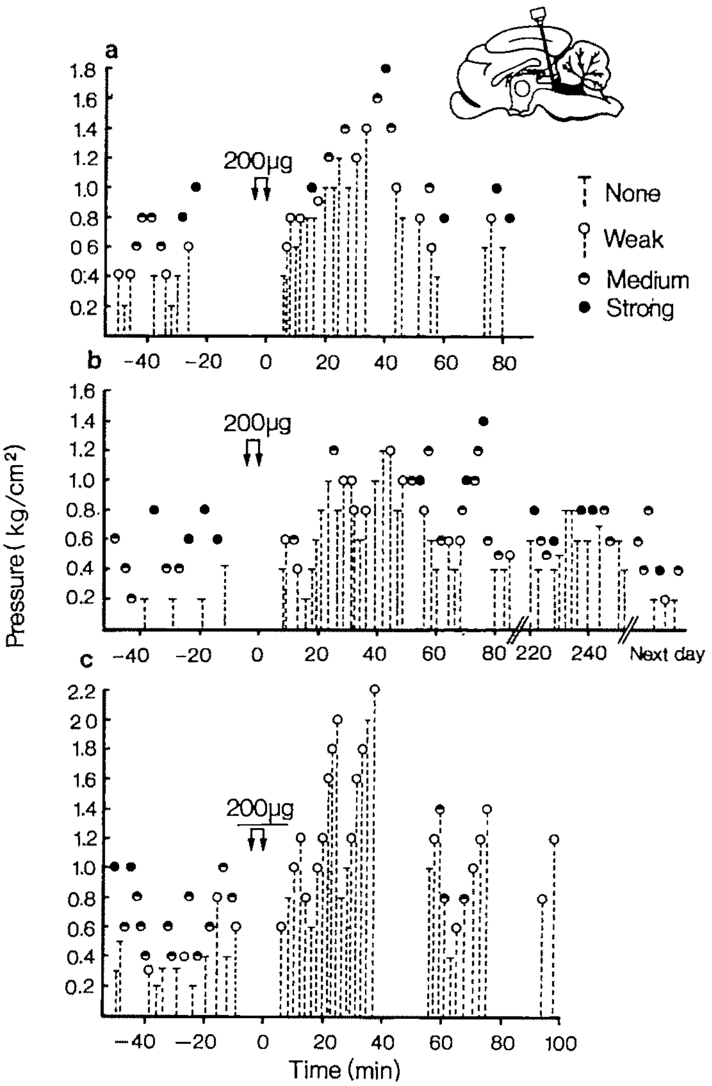


Figure 2 Responses in three cats to pressure exerted for 5 s on the root of the tail before and after infusion of 200 µg of morphine into the fourth ventricle. In this and Figures 3 and 4, the ordinates give the pressure in kg/cm² measured by a Bourdon gauge in the device used which is not identical, but proportional to that exerted on the tail (see Methods section).

saulting and/or of strong arching and vigorous struggling, of strong biting of the pincher or the attached tubing, and continued biting after the pressure was removed, and sometimes of miaowing or hissing and salivation as well.

A medium response suggested definite pain. It consisted of strong arching as the main or sole reaction, or of quickly getting up and rapidly moving about, or of some struggling with or without an attempt to remove the pincher, or of mild biting of the pincher or attached tubing, but with little or no struggling, or of a single miaow. Sometimes it was difficult to decide between a medium and a strong, or between a medium and a weak response.

In the course of several experiments a medium and a strong response became associated, particularly after morphine administration, with tachypnoea, mydriasis and the appearance or intensification of shivering which outlasted the 5 s period of increased pressure exerted on the tail for a minute or two. The morphine used was the sulphate and all values given in the text refer to the salt.

## Results

### *Infusion into the fourth ventricle and into the subarachnoid space beneath the ventral surface of the brain stem*

*Fourth ventricle.* Infusion of 200 µg of morphine into the fourth ventricle produced strong analgesia. The effect was not the result of absorption of morphine into the blood stream, because an intravenous injection of double the dose did not produce analgesia. The analgesia produced on infusion into the fourth ventricle began to develop within 5 to 10 min and became maximal within 15 to 40 min after the infusion, but varied in intensity and duration from experiment to experiment. This is illustrated by the three experiments of Figure 2.

The top record (Figure 2a) is from an experiment in which the analgesic effect was maximal after about 30 min, when the pressure exerted on the root of the tail could be increased to as much as 1.4 kg/cm$^2$ without producing more than a weak reaction. Before the infusion this happened when the pressure was not greater than 0.4 kg/cm$^2$. The middle record (b) is from an experiment in which some remnant of the analgesic effect was present 4 h after the infusion, and the bottom record (c) illustrates an experiment in which the analgesia produced was particularly strong so that a pressure of 2.2 kg could be applied without producing more than a weak response. This experiment illustrates another feature often encountered during morphine analgesia; a weak response was elicited whether the pressure applied was only 0.8 kg/cm$^2$ or as much as 2.2 kg/cm$^2$. Before a morphine infusion the difference in pressure required to change a weak to a medium or a medium to a strong response was relatively small, such a steep gradient often no longer pertained during the morphine analgesia.

Another feature of the morphine analgesia not evident from the scoring used was the delay of the reaction. Before the morphine infusion, a medium or strong reaction as well as a weak reaction occurred usually immediately the pressure was raised, whereas during the morphine analgesia the cat often did not react immediately, but only during the last second or two of the 5 s period of increased pressure, or at the very end of this period, or even a second or two later.

In two experiments in which 100 µg of morphine were infused the pressure which would produce not more than a mild response was, before the infusion, 0.2 kg/cm$^2$ in the one and 0.6 kg/cm$^2$ in the other experiment; in both it increased after the infusion to 1 kg/cm$^2$. And in three experiments in which 50 µg were infused this pressure increased from 0.4 kg/cm$^2$ in all three experiments before, to 0.6, 0.8 and 1.2 kg/cm$^2$ respectively after the infusion. Smaller doses than 50 µg were not tested, but the infusion of saline (40 µl) had no analgesic effect.

*Subarachnoid space beneath the brain stem.* Infusion of 200 µg of morphine into the subarachnoid space caudal to the pons produced strong analgesia, and the results obtained with any of the four methods which are illustrated in the diagrams B, C, D and E, of Figure 1 were essentially the same. Table 1 summarizes the results of 13 experiments, and for four of them (Nos 1, 7, 9 and 11) the development and disappearance of the analgesia is illustrated in Figure 3.

The analgesia produced on the infusion of morphine

**Table 1** Analgesia produced in 13 cats on infusion of 200 µg morphine into subarachnoid space caudal of pons

| | *Threshold pressure (kg/cm$^2$) on tail which produces no, or weak response* | | *Time (min) after morphine infusion* | | |
|---|---|---|---|---|---|
| *Cat. no.* | *Before morphine* | *After morphine* | *Beginning of analgesia* | *Maximal analgesia* | *Method used (see Figure 1)* |
| 1 | 0.3 | 0.8 | 1 | 7 | D |
| 2 | 0.3 | 1.0 | 1 | 17 | E |
| 3 | 0.3 | 1.0 | 6 | 33 | D |
| 4 | 0.4 | 1.0 | 1 | 6 | E |
| 5 | 0.2 | 1.2 | 6 | 16 | C |
| 6 | 0.4 | 1.2 | 3 | 9 | B |
| 7 | 0.4 | 1.4 | 6 | 30 | C |
| 8 | 0.6 | 1.4 | 3 | 10 | C |
| 9 | 0.3 | 1.6 | 5 | 28 | C |
| 10 | 0.4 | 1.6 | 2 | 19 | E |
| 11 | 0.6 | 2.0 | 3 | 12 | E |
| 12 | 0.8 | 2.2 | 6 | 25 | C |
| 13 | 0.8 | 2.6 | 5 | 16 | D |

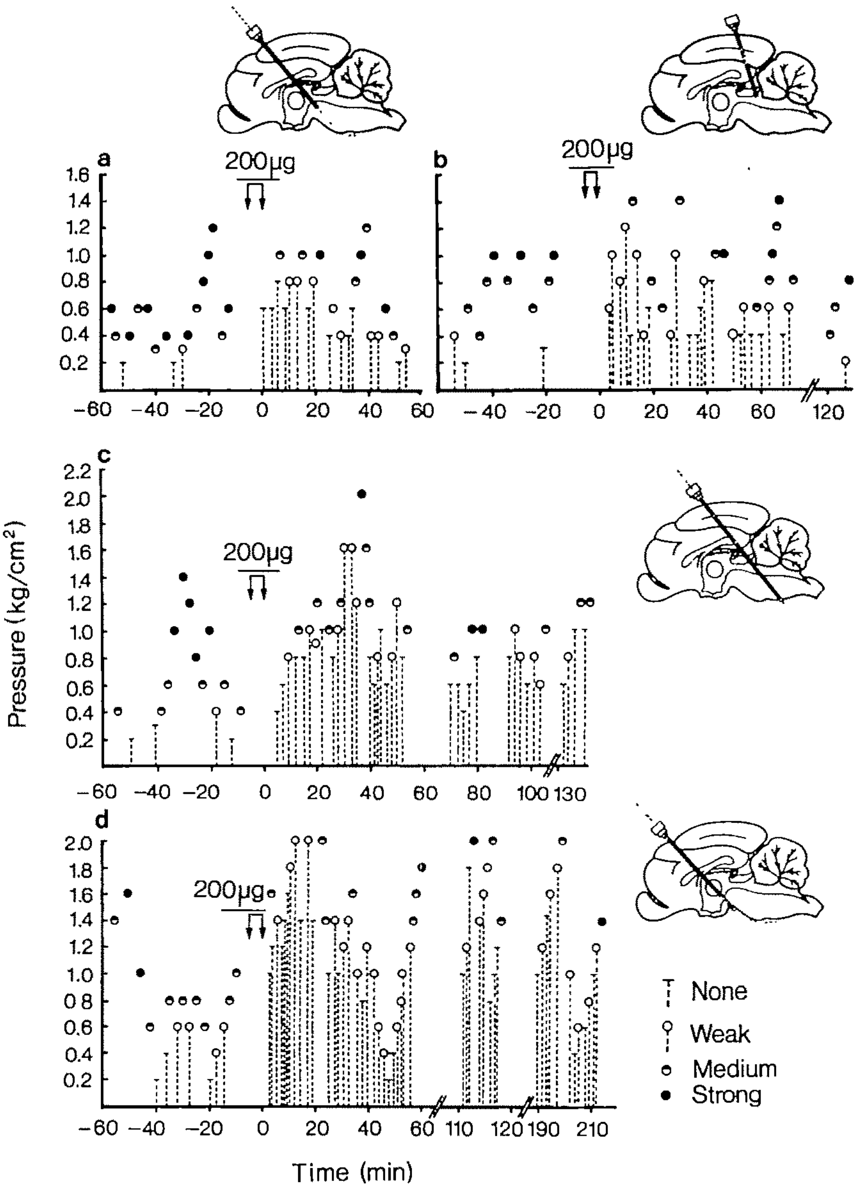


**Figure 3** Responses in four cats to pressure exerted for 5 s on the root of the tail before and after infusion of 200 µg of morphine into the subarachnoid space beneath the ventral surface of the brain stem using the methods D and B (top records, a and b), C (middle record, c), and E (bottom record, d) of Figure 1.

caudal to the pons often began earlier and developed more rapidly than on its infusion into the fourth ventricle. On infusion of 200 µg into the fourth ventricle no definite signs of analgesia were obtained when the tail pinch test was made earlier than 5 min after the infusion, but this happened in 7 out of the 13 experiments of Table 1, and in three of them signs of analgesia were already present 1 min after the infusion. Further, on infusion into the fourth ventricle, it took 20 to 40 min for the analgesia to develop fully whereas in 6 of the 13 experiments of Table 1 it took less than 15, and in three, even less than 10 minutes.

In experiment Nos 5 and 12 of Table 1 in which method C was used, the infusion of bromophenol blue at the end of the experiment resulted in deep staining of the needle tract within the brain stem. The infused morphine probably followed the same distribution. Nevertheless, an action on structures along the needle tract can be excluded even as a contributory factor in the morphine analgesia, because in two experiments (not included in the Table) in which method C was used the tip of the infusion needle had not pierced the ventral surface, but had stopped about 1 mm short of it, no analgesia developed after the morphine infusion. On subsequent bromophenol infusion, it was found that the dye had not entered the subarachnoid space but had deeply stained the whole needle tract in the brain stem and at its lower end around the tip of the cannula a cavity had formed which was deeply stained as well.

Figure 3 illustrates four experiments of Table 1 in which the infusion of 200 µg of morphine into the subarachnoid space caudal to the pons produced analgesia of different degree and duration. For each experiment a different method of infusion (either B, C, D or E of Figure 1) was used. In the experiment of Figure 3a, analgesia had disappeared within 50 min; in that of Figure 3b it was maximal within 9 min, and had not fully disappeared within 70 min; in the experiments c and d analgesia persisted even longer, for over 2 h in c and for over 3.5 h in d. These two experiments illustrate another feature encountered in that analgesia after having diminished, sometimes increased again.

In one experiment in which method E was used, the needle had pierced the dura and glided subdurally in the region of the trapezoid bodies. No analgesia developed on infusion of 200 and 400 µg morphine. In two experiments, one of which is illustrated in Figure 4a, the infusion of 200 µg of morphine was into the fossa interpeduncularis (method F of Figure 1). No analgesia developed.

*Other effects.* During or shortly after the infusions of 200 µg of morphine into the fourth ventricle or into the subarachnoid space caudal to the pons, the vessels of the pinna dilated and the ears became red and hot. This skin vasodilatation persisted for up to 3 hours. Another long lasting effect was midriasis. On infusion into the fourth ventricle it occurred within a few minutes, on infusion caudal to the pons 15 to 60 min after the infusion. Other effects not regularly observed were salivation, retching, vomiting, shivering and in one experiment on infusion caudal to the pons, tachypnoea and panting.

The behavioural changes brought about by the infusions into the fourth ventricle and caudal to the pons consisted of an initial period of quietness with or without signs of stupor and catalepsy followed by a characteristic state of hyperexcitability. In addition, the cats became affectionate and playful.

Either during or shortly after the infusions, the cats became quiet. A previously lively cat no longer moved about, but assumed a low sitting position. Some of the cats became stuporous. Slight cataleptic features were observed in one cat on infusion into the fourth ventricle and pronounced catalepsy lasting for over 2 h developed in two cats (Nos 11 and 13 of Table 1) on infusion caudal to the pons. The two cats could be put in abnormal positions which they retained without struggling for 20 to 30 s, for instance, when they were placed in an erect posture with the forepaws on the rung of an inverted stool. Nevertheless when the forepaws dropped from the rung the cats moved away in a well co-ordinated manner showing no signs of ataxia.

Before the state of hyperexcitability developed the cats were sitting upright with a startled look: the eyes wide open, pupils dilated, no blinking and the ears held sharply erect. This condition in which the cats were staring vacantly, lasted between a few minutes and over half an hour, and the the cats began to make, every few seconds, abrupt side-to-side movements of the head, and less frequently, rapid, short-lasting circling movements which apparently occurred spontaneously. But they could be triggered off by sudden noises and were provoked each time the cats were touched. Yet the cats were not frightened. In between the circling movements they resumed the upright sitting position with a wide-eyed vacant stare. The abrupt side-to-side movements of the head and the short-lasting rapid circling movements continued usually for over 2 hours. On infusion into the fourth ventricle the condition of hyperexcitability developed only rarely, but the preceding state in which the cats sat upright with a wide-eyed vacant stare was always present. Between the state of quietness and hyperexcitability, or when the hyperexcitability was wearing off, or in both conditions, the cats were affectionate and playful.

### *Injections into the cisterna magna*

Morphine produced analgesia on injection into the cisterna magna but the doses required were larger than on infusion into the fourth ventricle or on infusion into the subarachnoid space caudal to the pons. However, the injections were made in a larger volume, in 0.1 ml washed in by another 0.1 ml as compared to 40 µl the volume used for the infusions of morphine.

With 400 µg of morphine injected into the cisterna there was just an indication of analgesia, but with 800 µg a strong and long-lasting analgesia ensued, as illustrated by the experiment shown in Figure 4b. In this experiment the tail pinch test could not be applied during the first 10 min after the injection because of frequent bouts of scratching movements which morphine is known to produce on injection into the cisterna and into the cerebral ventricles. The scratching movements are attributed to an action on structures near the dorso-lateral surface of the upper cervical cord (Mehes, 1939, Königstein, 1939; Banerjee, Feldberg & Lotti, 1968). After the first 10 min the bouts of scratching movements occurred

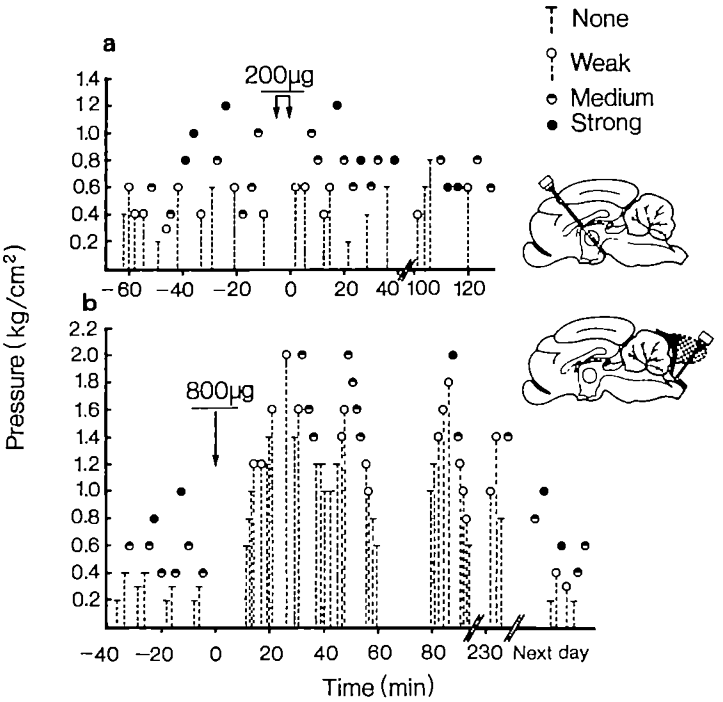


**Figure 4** Responses in two cats to pressure exerted for 5 s on the root of the tail. (a) Before and after infusion of 200 µg of morphine into the fossa interpeduncularis using method F of Figure 1. (b) Before and after injection of 800 µg of morphine into the cisterna magna.

less frequently allowing tests to be made in between, and after another 10 to 20 min they occurred at long intervals and in between the cats became quiet so that there was scarcely any interference with the tests. At this stage the pupils were dilated and the ears were hot and red. During the initial stage of frequent bouts of scratching movements there was tachypnoea and sometimes there were short periods of panting. In one experiment in which bouts of scratching movements were less violent and occurred less frequently during the first 10 min after the injection, the tail pinch test was applied every minute from the third minute after the injection; the first signs of analgesia occurred in the eleventh minute.

From results obtained on injection of bromophenol blue into the cisterna magna it is evident that even during the violent bouts of scratching substances injected into the cisterna do not enter the cerebral ventricles. Bromophenol blue injected into the cisterna magna also produces violent bouts of scratching movements (Feldberg & Fleischhauer, 1960). At the end of the experiment shown in Figure 4b, 0.2 ml of a 0.8% bromophenol blue was injected into the cisterna. After a few minutes the bouts of scratching movements became so violent that the cat was anaesthetized, 10 min after the injection, with intraperitoneal pentobarbitone sodium. The scratching movements continued and the cat was killed after another 10 minutes. Post mortem, no dye was found in the cerebral ventricles; there was no staining of the floor of the fourth ventricle, of the walls of the aqueduct, third and lateral ventricles. But the dorsal and ventral surface of the brain stem and cervical cord were deeply stained, and the dye had spread along the cerebellum and cerebral cortices and part of their surfaces were well stained. A similar staining had previously been observed with smaller amounts of bromophenol blue injected into the cisterna magna of anaesthetized rabbits (Banerjee, Burks, Feldberg & Goodrich, 1968).

## Discussion

The present experiments on cats show that morphine produces analgesia when introduced into the liquor space in doses too small to be effective on intravenous injection. The effect could therefore not have been produced after absorption into the blood stream. Instead, the morphine must have acted on structures reached from the liquor space.

The analgesia occurred not only on infusion of morphine into the fourth ventricle, but also on its infusion or injection into the subarachnoid space, either into the space beneath the brain stem caudal to the pons, or into the cisterna magna. In both circumstances, the structures acted upon must have been reached from the subarachnoid space since the morphine would not have entered the cerebral ventricles. The fact that somewhat larger doses were required to produce analgesia on injection into the cisterna than on infusion beneath the brain stem, suggests an action on structures near its ventral surface because on the way to this surface morphine injected into the cisterna becomes more diluted with c.s.f. than when infused directly beneath it. This surface would also readily be reached, without much dilution, by morphine infused into the fourth ventricle, because of the communication through the lateral recesses with the subarachnoid space.

The ventral surface of the brain stem would therefore appear to be the site at which morphine acts in all three circumstances when producing analgesia. It is possible that morphine infused into the fourth ventricle acts on an additional site in the floor of this ventricle although no evidence to support the existence of such a site was obtained by comparing the times it took for the first signs of analgesia to appear and for full analgesia to develop. These times were often shorter on infusion into the space beneath the ventral surface than on infusion into the fourth ventricle.

The situation is different when morphine is injected into a lateral or into the third ventricle when it may act not only on the ventral surface of the brain stem but also on the grey matter of the wall of the third ventricle and on the periaqueductal grey since microinjections of morphine into these regions were found to produce analgesia (Tsou & Tang, 1962; Jacquet & Lajtha, 1973; Pert & Yaksh, 1974). In fact, it was recently found that on infusion of morphine into the third ventricle, the analgesia produced was more pronounced than on infusion into the fourth ventricle or into the subarachnoid space caudal to the pons (Dashwood & Feldberg, unpublished experiments). With the methods used in the present experiments, however, the third ventricle and aqueduct are not reached by the morphine.

What then are the structures on or near the ventral surface of the brain stem on which morphine may act when producing analgesia? One mechanism by which morphine is thought to produce analgesia is by activating a descending inhibitory pathway which originates in the raphe nuclei, is tryptaminergic, projects to the spinal cord and there exerts an inhibitory action on the small-diameter afferent fibres conveying pain impulses. There is abundant evidence for this mechanism of action. Morphine analgesia is reduced or abolished by substances which selectively deplete tryptaminergic neurones of their 5-hydroxytryptamine (5-HT), like *p*-chlorophenylalanine (Tenen, 1968; Fenessy & Lee, 1970; Samanin, Gomulka & Valzelli, 1970; Görlitz & Frey, 1972; Vogt, 1974) and 5,6-dihydroxytryptamine (Genovese, Zonta & Mantegazza, 1973; Diaz, Ellison & Masuoka, 1974; Vogt, 1974). Furthermore, electrical stimulation of the raphe nuclei and of many points of the periaqueductal grey in their vicinity elicit analgesia which is blocked by 5-HT depletion produced by *p*-chlorophenylalanine (Reynolds, 1969; Akil & Mayer, 1972; Mayer & Liebeskind, 1974; Mayer, Wolfle, Akil, Carder & Liebeskind, 1974; Akil & Liebeskind, 1975; Proudfit & Anderson, 1975), whereas lesions of the raphe nuclei which lead to 5-HT depletion block morphine analgesia (Redjemi, Oliveras, Guilbaud & Besson, 1974; Proudfit & Anderson, 1975).

It is doubtful that substances injected into the cerebral ventricles reach the raphe nuclei. Otherwise a substance which depletes the 5-HT stores of the brain should also exert this effect on the 5-HT of the raphe nuclei when injected intraventricularly. Yet, 5,6-dihydroxytryptamine given into a lateral ventricle while reducing the 5-HT of the spinal cord by nearly 90% had scarcely any effect on the mid brain 5-HT (Baumgarten, Evetts, Holman, Iversen, Vogt & Wilson, 1972; Vogt, 1974). This was in contrast to the effect of *p*-chlorophenylalanine given systematically. It depleted not only the 5-HT of the spinal cord, but also that of the midbrain (Vogt, 1974). A convincing explanation of this difference in the effect of the tryptamine derivative would be that it does not reach the raphe nuclei on intraventricular injection, but reached its descending tryptaminergic fibres on their way to the spinal cord. The site reached and acted upon by the 5,6-dihydroxytryptamine would then appear to be at the ventral surface of the brain stem lateral to the pyramids, because these descending tryptaminergic fibres run to the ventral surface of the medulla and form a narrow distinct band lateral to each pyramid. This was shown by Nobin, Baumgarten, Bjökland, Lachenmayer & Stenevi (1973). An additional observation favouring this view was made by Vogt (1974). In her rats a permanent brownish discolouration indicating the spread of 5,6-dihydroxytryptamine was found following its injection into a lateral ventricle 'on the surface of the third ventricle, the aqueduct and the anterior part of the fourth ventricle' but not on its posterior part which is evidence that it had passed through the lateral recesses into the subarachnoid space. From these various findings it would appear that 5,6-dihydroxytryptamine has the ability to deplete 5-HT stores when acting on fibres of tryptaminergic neurones.

Morphine, too, would not reach the raphe nuclei when infused into the fourth ventricle, or into the subarachnoid space beneath the brain stem, or injected into the cisterna magna. But in all three circumstances, it would reach the ventral surface of the brain stem. Therefore if the morphine analgesia obtained in the present experiments is due to activation of the tryptaminergic descending inhibitory pathway, the possibility has to be considered that it, too, acts not on synapses or cells, but on nerve fibres belonging to tryptaminergic neurones. This possibility would be compatible with all observations made in favour of the theory that morphine analgesia involves activation of a descending inhibitory tryptaminergic pathway arising from the raphe nuclei.

If activation of a descending inhibitory tryptaminergic pathway is also responsible for the analgesia produced by morphine applied systemically, and all the evidence with *p*-chlorophenylalanine and with lesions of the nucleus raphe magnus for the role of such a descending inhibitory pathway in morphine analgesia was obtained with the morphine applied systemically, then its action under this condition may be on the raphe nuclei themselves. The finding that 5,6-dihydroxytryptamine merely reduced the analgesia of morphine given subcutaneously but did not abolish it is readily explained on the assumption that not all descending fibres from this raphe nucleus were reached and affected by the 5,6-dihydroxytryptamine injected intraventricularly with the result that the spinal cord 5-HT was greatly reduced but not fully depleted as after systemic application of *p*-chlorophenylalanine. As pointed out by Vogt (1974), activation of the remaining intact tryptaminergic fibres may have been sufficient to produce analgesia.

But even on systemic application, the analgesic effect of morphine may in part, at least, be due to an action not on the raphe nuclei themselves, but on their descending tryptaminergic fibres. For instance, in the experiments of Satoh & Takagi (1971) described in the introduction, intravenous morphine (4 mg/kg) acted caudal to the trapezoid bodies within a 4 mm long block of brain stem tissue which at most included but the very distal remnants of raphe nuclei. An action on them may be sufficient to account for the inhibitory effect morphine produced in these experiments on afferent pain impulses. This possibility cannot be excluded. But there is the other possibility of an action on the descending tryptaminergic fibres as they emerge on the ventral surface in this region of the brain stem, which is penetrated here by numerous small arteries and thus provided with an abundant blood supply.

The concept that morphine, like 5,6-dihydroxytryptamine, acts on nerve fibres may apply only to fibres of tryptaminergic neurones, because of their peculiar property of becoming activated for a long time by morphine. Non-myelinated fibres of non-tryptaminergic neurones may not share this morphine sensitivity. For instance, Kosterlitz & Wallis (1964) found no effect of morphine on the C fibres of peripheral nerves, but they looked mainly for an inhibitory effect. If sensitivity to morphine is peculiar to fibres of tryptaminergic neurones, other effects of morphine, apart from analgesia, may well be accounted for by a synaptic action which may even apply to the analgesia produced by large doses of morphine on the spinal cord. In the experiments of Satoh & Takagi (1971), cervical cord transsection did not prevent large doses of morphine (8 to 10 mg/kg intravenously) from abolishing the afferent volleys in the cord produced on stimulation of the splanchnic nerves. The descending tryptaminergic fibres in the cord were not previously put out of action in these experiments, either by lesion of the raphe nucleus magnus, by *p*-chlorophenylalanine or by 5,6-dihydroxytryptamine. But if the effect was not dependent on the integrity of the descending tryptaminergic fibres in the cord, it could result from a synaptic action of morphine in the spinal cord.

P.K.D. was supported initially by the Wellcome Trust and later by the JKFE fund.

**References**

AKIL, H. & LIEBESKIND, J.C. (1975). Monoaminergic mechanisms of stimulation-produced analgesia. *Brain Research*, **94**, 279–296.

AKIL, H. & MAYER, D.J. (1972). Antagonism of stimulation-produced analgesia by p-CPA, a serotonin synthesis inhibitor. *Brain Research*, **44**, 692–697.

BANERJEE, U., BURKS, T.F., FELDBERG, W. & GOODRICH, CECILIE A. (1968). Temperature effects and catalepsy produced by morphine injected into the cerebral ventricles of cats. *Br. J. Pharmac. Chemother.*, **33**, 544–551.

BANERJEE, U., FELDBERG, W. & LOTTI, V.J. (1968). Effect on body temperature of morphine and ergotamine injected into the cerebral ventricles of cats. *Br. J. Pharmac. Chemother.*, **32**, 523–538.

BAUMGARTEN, H.G., EVETTS, K.D., HOLMAN, R.B., IVERSEN, L., VOGT, M. & WILSON, G. (1972). Effects of 5,6-dihydroxytryptamine on monoaminergic neurones in the central nervous system of the rat. *J. Neurochem.*, **19**, 1587–1597.

DEY, P.K. & FELDBERG, W. (1975a). Hyperglycaemia produced by drugs with analgesic properties introduced into the cerebral ventricles of cats. *Br. J. Pharmac.*, **54**, 163–170.

DEY, P.K. & FELDBERG, W. (1975b). Morphine analgesia—its main site of action? *J. Physiol., Lond.*, **248**, 40–41P.

DEY, P.K., FELDBERG, W. & WENDLANDT, SABINE (1975). Comparison of the hyperglycaemic effect of adrenaline and morphine introduced into the liquor space. *J. Physiol., Lond.*, **246**, 213–228.

DIAZ, J., ELLISON, G. & MASUOKA, D. (1974). Opposed behavioral syndromes in rats with partial and more complete central serotonergic lesions made with 5,6-dihydroxytryptamine. *Psychopharmacologia (Berl.)*, **37**, 67–79.

FELDBERG, W. & FLEISCHHAUER, K. (1960). Scratching movements evoked by drugs applied to the upper cervical cord. *J. Physiol., Lond.*, **151**, 502–517.

FELDBERG, W. & GUPTA, K.P. (1974). Morphine hyperglycaemia. *J. Physiol., Lond.*, **238**, 487–502.

FELDBERG, W., GUPTA, K.P., MILTON, A.S. & WENDLANDT, SABINE (1973). Effect of pyrogen and antipyretics on prostaglandin activity in cisternal c.s.f. of unanaesthetized cats. *J. Physiol., Lond.*, **234**, 279–303.

FENNESSY, M.R. & LEE, J.R. (1970). Modification of morphine analgesia by drugs affecting adrenergic and tryptaminergic mechanisms. *J. Pharm. Pharmac.*, **22**, 930–935.

GENOVESE, E., ZONTA, N. & MANTEGAZZA, P. (1973). Decreased antinociceptive activity of morphine in rats pretreated intraventricularly with 5,6-dihydroxytryptamine, a long-lasting selective depletor of brain serotonin. *Psychopharmacologia (Berl.)*, **32**, 359–364.

GÖRLITZ, B.-D. & FREY, H.-H. (1972). Central monoamines and antinociceptive drug action. *Eur. J. Pharmac.*, **20**, 171–180.

HERZ, A., ALBUS, K., METYS, J., SCHUBERT, P. & TESCHENMACHER, Hj. (1970). On the central sites for the antinociceptive action of morphine and fontanyl. *Neuropharmacology*, **9**, 539–551.

JACQUET, Y.F. & LAJTHA, A. (1974). Paradoxical effects after microinjection of morphine in the periaqueductal gray matter in the rat. *Science*, **185**, 1055–1057.

KÖNIGSTEIN, H. (1939). Über Localisationsversuche des durch Morphium ausgelösten 'Kratzwerkes' im Zentral nerven system. *Arch. int. Pharmacodyn.*, **62**, 1–13.

KOSTERLITZ, H.W. & WALLIS, D.I. (1964). The action of morphine-like drugs on impulse transmission in mammalian nerve fibres. *Br. J. Pharmac. Chemother.*, **22**, 499–510.

MAYER, D.J. & LIEBESKIND, J.C. (1974). Pain reduction by focal electrical stimulation of the brain: an anatomical and behavioral analysis. *Brain Research*, **68**, 73–93.

MAYER, D.J., WOLFLE, T.L., AKIL, H., CARDER, B., & LIEBESKIND, J.C. (1971). Analgesia from electrical stimulation in the brainstem of the rat. *Science*, **174**, 1351–1354.

MEHES, J. (1939). Experimentelle Untersuchungen über den Juckreflex am Tier. 2 to Mitteilung-Auslösen heftiger Juckanfälle bei der Katze durch intracisternale Injectionen von Morphium und einige seiner Derivate. *Naunyn Schmiedebergs Arch. exp. Path. Pharmak.*, **188**, 650–656.

NOBIN, A., BAUMGARTEN, H.G., BJÖRKLUND, A., LACHENMAYER, L. & STENEVI, U. (1973). Axonal degeneration and regeneration of the bulbospinal indolamine neurons after 5,6-dihydroxytryptamine treatment. *Brain Research*, **56**, 1–24.

PERT, A. & YAKSH, T. (1974). Sites of morpine induced analgesia in the primate brain: relation to pain pathways. *Brain Research*, **80**, 135–140.

PROUDFIT, H.K. & ANDERSON, E.G. (1975). Morphine analgesia: blockade by raphe magnus lesions. *Brain Research*, **98**, 612–618.

REDJEMI, E., OLIVERAS, J.-L., GUILBAUD, G. & BESSON, J.-M. (1974). Analgésie induite par la stimulation du noyau central inférieur du raphé chez le chat. *C.R. Acad. Sci. (Paris)*, **279**, 1105–1107.

REYNOLDS, D.V. (1969). Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science*, **164**, 444–445.

RUSSELL, W.J. & TATE, M.A. (1975). A device for applying nociceptive stimulation by pressure. *J. Physiol., Lond.*, **248**, 5–7P.

SAMANIN, R., GUMULKA, W. & VALZELLI, L. (1970). Reduced effect of morphine in midbrain raphe lesioned rats. *Eur. J. Pharmac.*, **10**, 339–343.

SATOH, M. & TAKAGI, H. (1971). Enhancement by morphine of the central descending inhibitory influence on spinal sensory transmission. *Eur. J. Pharmac.*, **14**, 60–65.

TENEN, S.S. (1968). Antagonism of the analgesic effect of morphine and other drugs by *p*-chlorophenylalanine, a serotonin depletor. *Psychopharmacologia (Berl.)*, **12**, 278–285.

TSOU, K. & TANG, Ch. (1962). Analgesic effect of intraventricular or intracerebral microinjection of morphine. (English abstract.) *Acta physiologica Sinica*, **25**, 119–128.

VOGT, MARTHE (1974). The effect of lowering the 5-hydroxytryptamine content of the rat spinal cord on analgesia produced by morphine. *J. Physiol., Lond.*, **236**, 483–498.

*(Received April 5, 1976)*

*Br. J. Pharmac.* (1976), **58**, 395–400

# ACTIONS OF CARBON TETRACHLORIDE, HEXACHLOROETHANE AND THE PRODUCTS OF THEIR METABOLISM IN SHEEP ON *Fasciola hepatica*

A.L. BARTLET
Department of Veterinary Pharmacology, Edinburgh, EH9 1QH

1 An *in vitro* preparation of *Fasciola hepatica* is described which responded to electrical stimulation with tetanic spasms. Both carbon tetrachloride (20–500 nl/ml), and its metabolite chloroform (50–1000 nl/ml), produced contractions in the preparation which extinguished the responses to electrical stimulation. It is suggested that the spasmogenic action of carbon tetrachloride and its metabolite may contribute to the fasciolifugal action of the drug.

2 Hexachloroethane, another fasciolifuge, had very little effect in the preparation. However, pentachloroethane and tetrachloroethylene, the main products of the metabolism of hexachloroethane in sheep, were potent spasmogens in preparations of *Fasciola hepatica*. Pentachloroethane was about twice as potent as carbon tetrachloride.

3 Tetrodotoxin (2 µg/ml) did not antagonize the responses of the preparation to electrical stimulation or carbon tetrachloride.

## Introduction

It has been shown that carbon tetrachloride is without effect on the survival time of *Fasciola hepatica in vitro* (Stephenson, 1947; Thorpe, 1967). The mode of the fasciolifugal action of carbon tetrachloride is unknown, and may be an indirect effect mediated by metabolic products of the drug or materials produced by the liver when it is affected by the drug (Alexander & MacDonald, 1960; Kondos & McClymont, 1965; Khalidi & Zaki, 1969). In sheep considerable amounts of carbon tetrachloride are metabolized to chloroform, and the main products in the metabolism of hexachloroethane, another fasciolifuge, are pentachloroethane and tetrachloroethylene (Fowler, 1969; 1970).

According to Chance & Mansour (1949) both carbon tetrachloride and hexachloroethane contract an *in vitro* preparation of *Fasciola hepatica*. This observation is of interest as spasm of the mature liver flukes might cause them to leave their location in the bile ducts. This would be in accord with the effect of therapeutic doses of carbon tetrachloride and hexachloroethane which eliminate the liver flukes in bile ducts and not those sequestered in liver parenchyma (Boray & Happich, 1968). In the present experiments the effects of carbon tetrachloride and hexachloroethane on *Fasciola hepatica* have been re-examined and compared with those of chloroform, pentachloroethane and tetrachloroethylene.

## Methods

### *Preparations of* Fasciola hepatica

*Fasciola hepatica* was obtained from the bile ducts of infested liver from cattle or sheep before the organ had cooled to room temperature. Each liver fluke was washed and subsequently placed in a conical flask (25 ml) containing Ringer solution at 32°C. Viable liver flukes were motile for at least 24 h, and preparations were made from them within 2 h of collection.

Fine thread was passed through the body wall posterior to the ventral sucker and close to the caudal end of the animal. The liver fluke was attached, oral sucker downwards, to the electrode assembly (Figure 1), which was placed in an organ bath (5 ml) filled with Ringer solution maintained at 37°C and gassed with 5% $CO_2$ in $O_2$. The preparation relaxed and started rhythmic activity within 10 min, the activity being recorded on smoked paper with an isotonic lever, load 500 mg, which magnified responses twenty times. In most experiments the preparation was subsequently impaled with the platinum electrode between the oral and ventral suckers. These preparations were quiescent. In some experiments the preparations were stimulated with square wave pulses (5 ms, 30 Hz,

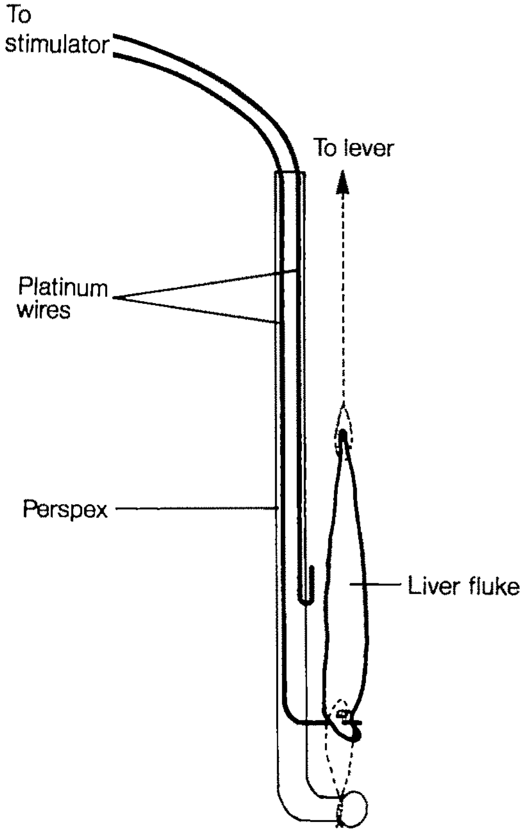


**Figure 1** Electrode assembly for stimulation of *Fasciola hepatica.*

25 V) delivered from a Bell (1968) stimulator for 20 s, the interval between trains of stimuli being 16 minutes. Drugs were washed from the organ bath by overflow.

### *Chemicals*

A Ringer solution of the composition described by Beretta & Locatelli (1968) was made with Analar salts and de-ionized water (composition (mM): NaCl 154.00, KCl 5.36, $CaCl_2$ 0.54, $NaHCO_3$ 5.95 and glucose 2.78). Hexachloroethane (Technical, BDH) was recrystallized from ethanol to constant melting point, 186°C uncorrected (sealed tube). Tetrachloroethylene and pentachloroethane (Technical, BDH) were purified by distillation, collecting the fractions at 119°C and 156°C (uncorrected), respectively. Other drugs were carbon tetrachloride (Analar, BDH), chloroform (Analar, BDH) and tetrodotoxin (Sigma). *n*-Heptane (Laboratory Reagent grade, BDH) was washed with 0.1 M NaOH, 0.1 M HCl and three times with distilled water.

The halogenated hydrocarbons were dissolved in olive oil B.P. (Evans Medical Ltd) and the solutions emulsified by trituration with acacia powder (BDH) and de-ionized water in a pestle and mortar. The primary emulsion contained oil, water and gum in the proportions 3:2:1, and was diluted with Ringer solution before testing on preparations of *Fasciola hepatica*. The droplet size (mean ± s.e. mean) in six emulsions as measured by microscope stage micrometer was $15.5 \pm 0.7$ µm.

Chloroform and carbon tetrachloride were estimated in the emulsions by gas/liquid chromatography as a significant amount of chloroform was lost in emulsification. Emulsion (1 ml) and heptane (4 ml) were shaken by hand in a glass stoppered centrifuge tube for 90 s, and the layers separated by centrifugation at 900 *g* for 10 min in an MSE Mistral 2L centrifuge at −10°C. The heptane extract was poured off the frozen residue into another tube and the residue re-extracted with heptane (4 ml).

### *Gas/liquid chromatography*

The chromatographic column was prepared according to a modification of the method described by Street (1969), who kindly supplied the packing materials. A 6 ft length of stainless steel tubing (i.d. 2.2 mm) was coiled into a helix and conditioned by heating in a Gallenkamp muffle furnace at 600°C for 16 hours. After cooling, chloroform (50 ml) and air were pulled through the tubing. A sintered steel plug was pressed into the column exit and the column packed with a preparation of Chromosorb G coated with silicone gum rubber (SE 52), tapping the tubing whilst sucking in the preparation at a water pump. The column was heated at 420°C for 3 h to make the inner metal surface unreactive. It was then emptied and repacked with a preparation of silanised Chromosorb G coated with PEG (Carbowax 400). A sintered Teflon plug was pressed into the column entrance and swagelock ferrules and compression nuts fitted on the steel tubing for attachment to a Perkin-Elmer Model F11 chromatograph.

The column temperature was adjusted to 85°C, the injector and detector temperatures being about 50° higher. The flow of carrier gas ($N_2$) was 45 ml/minute. Heptane extracts and solutions (1 to 5 µl) were injected with a micro-syringe fitted with a 3-inch needle and the signal from the electron capture detector recorded on a Servoscribe recorder (5 mV full scale deflection). The electron capture detector was sensitive to pl of choroform and carbon tetrachloride and a linear dose-response relationship was obtained with response expressed as peak height times width at half-height.

## Results

Preparations of *Fasciola hepatica* which were not impaled with an electrode exhibited irregular rhythmic

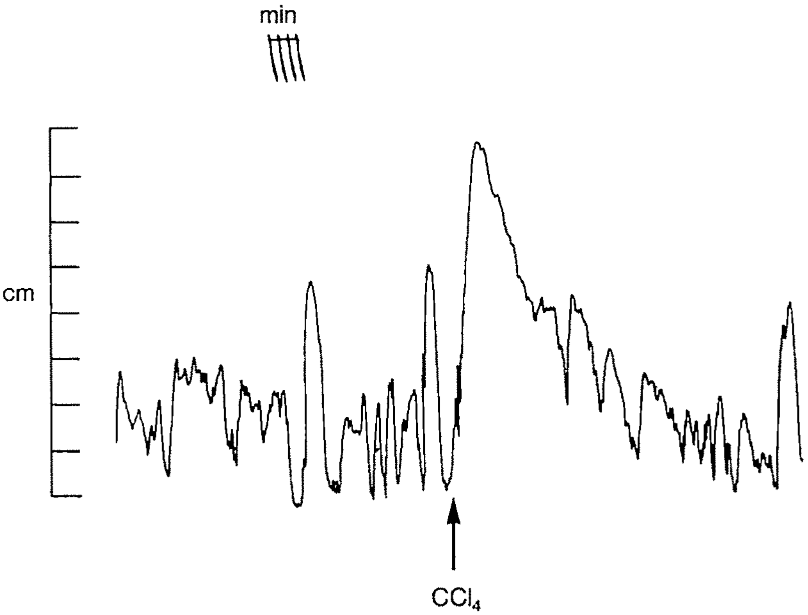


**Figure 2** *Fasciola hepatica* preparation. At the arrow carbon tetrachloride was added to the organ bath to produce a concentration of 100 nl/ml bathing solution. Although the preparation was not washed with fresh Ringer solution it resumed spontaneous rhythmic movements when the response to the drug subsided.

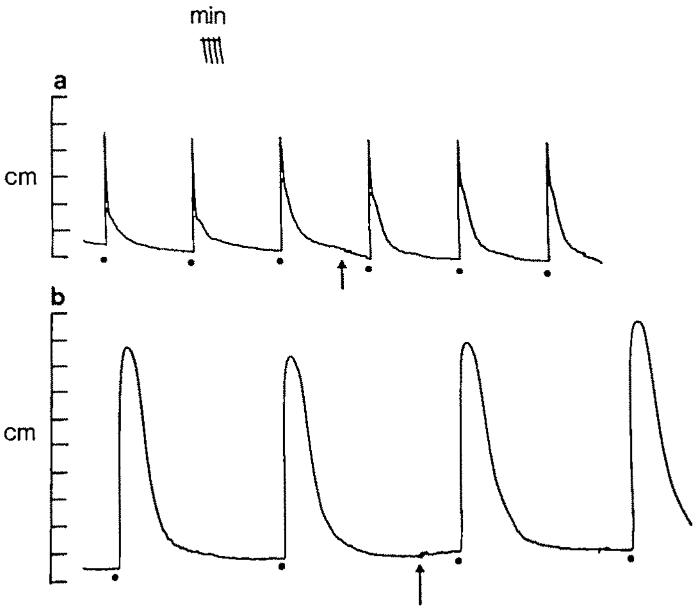


**Figure 3** Two preparations of *Fasciola hepatica* impaled with electrodes. In (a) the dots mark stimulation (5 ms, 30 Hz, 25 V) for 20 s and in (b) exposure to carbon tetrachloride (100 nl/ml) for 3 minutes. In both experiments tetrodotoxin (2 μg/ml) was present in the bathing solution from the arrow to the end of the trace. Tetrodotoxin did not affect the responses to electrical stimulation or carbon tetrachloride.

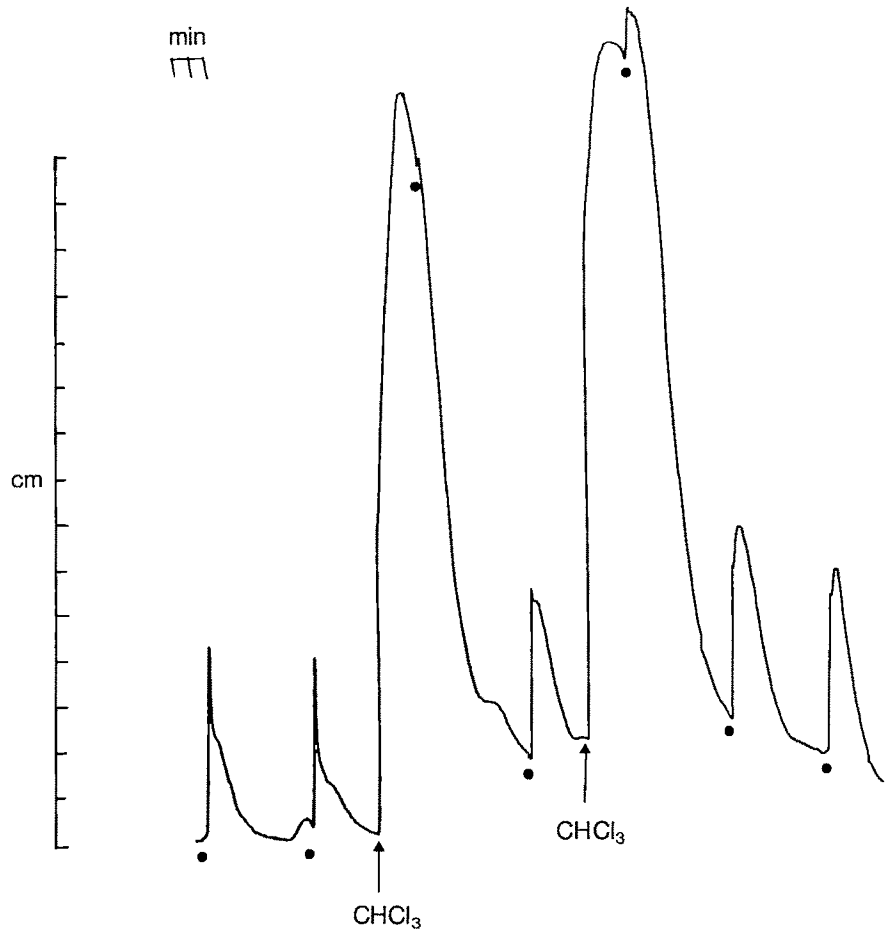


**Figure 4** *Fasciola hepatica* preparation. The dots mark stimulation (5 ms, 30 Hz, 25 V) of the preparation for 20 s and the arrows the addition of chloroform (200 nl/ml) to the bathing solution for 4 minutes. Chloroform produced large contractions which extinguished responses to electrical stimulation.

movements for several hours (Figure 2). On exposure to carbon tetrachloride (100 nl/ml) a slow contraction started after a delay of 1 to 2 min reaching a maximum in about 4 minutes. The response to the drug subsided and the rhythmic movements in the preparation resumed without washing the drug out of the organ bath. However, responses to drugs were irregular in preparations exhibiting spontaneous activity the height of the responses sometimes being ill-defined due to the spontaneous movements in the preparation.

In four experiments preparations were exposed to carbon tetrachloride (1000 nl/ml) for 30 min, the bubbling with 5% $CO_2$ in $O_2$ being stopped whilst the drug was present in the organ bath. This treatment produced a contracture. After removal of the drug from the bathing solution the preparations contracted normally to carbon tetrachloride (30 nl/ml) or electrical stimulation although spontaneous rhythmic activity did not recover. The treatment did not produce irreversible paralysis in the preparation as described by Chance & Mansour (1949).

The spontaneous rhythmic movements usually ceased when a preparation was impaled with an electrode, and this preparation responded regularly to electrical stimulation and carbon tetrachloride (Figure 3). Responses to single pulses were not obtained and trains of pulses (30 Hz, 25 V) were effective only when the pulse width was 1 ms or more. In the present experiments 20 s stimulations (5 ms, 30 Hz, 25 V) were delivered to the preparation which responded with slow contractions that continued to develop for several seconds after stimulation had stopped. Contractions to carbon tetrachloride at concentrations

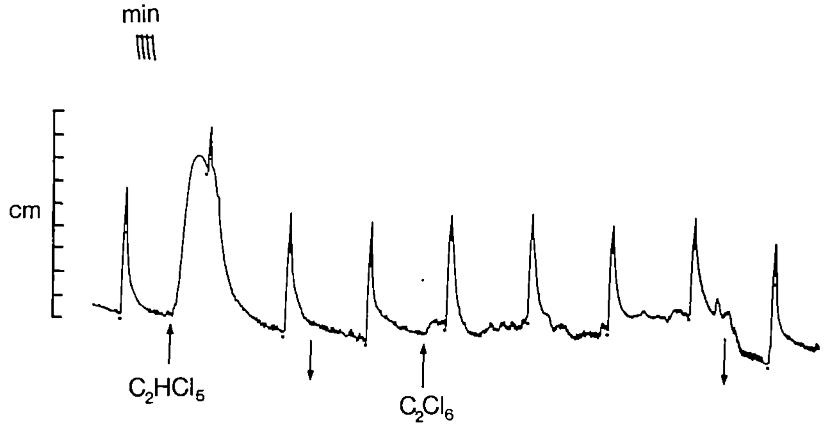


**Figure 5** *Fasciola hepatica* preparation. The dots mark stimulation (5 ms, 30 Hz, 25 V) of the preparation for 20 seconds. At the first and third arrows pentachloroethane ($C_2HCl_5$, 20 nl/ml) and hexachloroethane ($C_2Cl_6$, 1000 µg/ml) were added to the bathing solution, respectively, and at the second and fourth drugs were washed out of the organ bath. Pentachloroethane produced a large contraction which partly extinguished the response to electrical stimulation; hexachloroethane had little effect on the preparation.

of 20 to 500 nl/ml started within 10 s of exposure to the drug, and were reproducible when the preparation was exposed to the drug for 3 min at intervals of 20 minutes. Addition of tetrodotoxin, 2 µg/ml, to the bathing solution did not affect the responses to electrical stimulation or carbon tetrachloride (Figure 3).

Chloroform produced a contraction in preparations of *Fasciola hepatica* which started within 5 s of exposure to the drug (Figure 4), the response to chloroform being brisker than that to carbon tetrachloride. Both carbon tetrachloride and chloroform produced large contractions in the preparations which extinguished responses to electrical stimulation (Figure 4).

Six emulsions of chloroform and carbon tetrachloride were assayed by gas/liquid chromatography and tested on preparations of *Fasciola hepatica*. In these experiments the preparations were exposed to drugs for 3 min at intervals of 20 min and were not stimulated electrically. Emulsification produced a significant loss of chloroform ($P < 0.0001$) but not carbon tetrachloride ($P > 0.3$), the emulsions containing 70 ± 2% (mean ± s.e. mean) (6 expts) of the chloroform employed and 92 ± 8% (6) of the carbon tetrachloride. The spasmogenic activity of 1 µl carbon tetrachloride was found to be equivalent to that of 2.5 ± 0.6 µl chloroform (6 expts).

According to Chance & Mansour (1949) hexachloroethane, 100 µg/ml, stimulated preparations of *Fasciola hepatica*, and at higher concentration, 1000 µg/ml, produced paralysis. However, in the present experiments hexachloroethane (20 to 1000 µg/ml) had little effect in preparations responding to electrical stimulation, the effect of the drug (1000 µg/ml) shown in Figure 5 being typical of that in four preparations. Both pentachloroethane and tetrachloroethylene contracted preparations of *Fasciola hepatica*, the effect of pentachloroethane (20 nl/ml) being shown in Figure 5. The time course of the response to pentachloroethane was almost identical to that to carbon tetrachloride; that to tetrachloroethylene was somewhat slower. The spasmogenic activity of 1 µl carbon tetrachloride was matched by that of 0.56 ± 0.09 µl pentachloroethane and 3.46 ± 1.14 µl tetrachloroethylene (6 expts in each instance).

## Discussion

In the liver fluke preparation, tetrodotoxin did not block the contractions produced by electrical stimulation and carbon tetrachloride. Thus the component in the preparation which responded to these stimuli was qualitatively different from vertebrate nerve and striped muscle which are highly sensitive to tetrodotoxin. Spike generation in the musculature of the body wall of *Fasciola hepatica* may resemble that in plain muscle which is also insensitive to tetrodotoxin (Kuriyama, Osa & Toida, 1966). It is equally possible however, that the affinity of tetrodotoxin for Na channels in the membranes of nerve and muscle may be considerably less in *Fasciola hepatica* than in vertebrates.

The present experiments confirm that carbon tetrachloride contracts an *in vitro* preparation of *Fasciola hepatica*, the threshold concentration of the drug in an organ bath being 20–40 nl/ml. Furthermore chloroform contracted the preparation, being about a third as potent as carbon tetrachloride in this respect. Thus in infested ruminants the direct effect of carbon tetrachloride on *Fasciola hepatica* would summate with that of chloroform, its major metabolite.

In sheep carbon tetrachloride is rapidly absorbed from the rumen, the concentration in venous blood rising to 15–20 µg/ml following administration of 8 ml of the drug (Kondos & McClymont, 1961). The concentration of carbon tetrachloride in portal blood does not seem to have been estimated, but it is likely to be higher than that in the jugular vein. Kondos & McClymont (1965) found peak concentrations of 36 and 65 µg carbon tetrachloride per ml of sheep bile following oral administration of 2 and 4 ml of the drug, respectively, and Fowler (1970) detected 4–5 µg/ml after giving 3 ml of the drug.

Mature liver flukes ingest blood and produce extensive haemorrhages in the biliary tract (Sewell, Hammond & Dinning, 1968). Thus it seems probable that *Fasciola hepatica* is exposed to carbon tetrachloride at concentrations which are pharmacologically active following administration of the drug to sheep.

In the present experiments hexachloroethane had little effect in preparations of *Fasciola hepatica*, but pentachloroethane and tetrachloroethylene, the main products of the metabolism of hexachloroethane in sheep (Fowler, 1969), possessed marked pharmacological activity. Pentachloroethane was about twice as potent as carbon tetrachloride as a spasmogen in preparations of *Fasciola hepatica*. These observations suggest that spasmogenic activity may contribute to the fasciolifugal action of the chlorinated hydrocarbons.

I wish to thank Professor F. Alexander for his interest, Messrs D. Hall, K. Johnson and I. Warwick for technical assistance, Dr H. V. Street for advice on gas/liquid chromatography, Dr D. M. Woolley for the microscope stage micrometer, Mr R. K. Thomson for photography and the Veterinary officers and Meat Inspectors at the Slaughterhouses in Galashiels and Edinburgh for providing infested livers. Reprints are not available.

## References

ALEXANDER, F. & MacDONALD, D.C. (1960). The action of carbon tetrachloride on the sheep's liver. *Q. Jl. exp. Physiol.*, **45**, 12–17.

BELL, P.M.G. (1968). A new stimulator. *Br. J. Pharmac. Chemother.*, **32**, 435–436P.

BERETTA, C. & LOCATELLI, A. (1968). Inhibitory activity of 8β-carbobenzyl-oxyaminomethyl-1,6-dimethyl-10α-ergoline towards stimulant effects by 5-hydroxytryptamine and amphetamine on liver fluke, *Fasciola hepatica. J. Pharm. Pharmac.*, **20**, 744–745.

BORAY, J.C. & HAPPICH, F.A. (1968). Standardised chemotherapeutical tests for immature and mature *Fasciola hepatica* infections in sheep. *Aust. Vet. J.*, **44**, 72–78.

CHANCE, M.R.A. & MANSOUR, T.E. (1949). A kymographic study of the action of drugs on the liver fluke (*Fasciola hepatica*). *Br. J. Pharmac. Chemother.*, **4**, 7–13.

FOWLER, J.S.L. (1969). Some hepatotoxic actions of hexachloroethane and its metabolites in sheep. *Br. J. Pharmac.*, **35**, 530–542.

FOWLER, J.S.L. (1970). Carbon tetrachloride metabolism in sheep and in *Fasciola hepatica. Br. J. Pharmac.*, **39**, 599–607.

KHALIDI, A. & ZAKI, S.A. (1969). The mode of action of carbon tetrachloride on *Fasciola hepatica. Br. J. Pharmac.*, **36**, 253–256.

KONDOS, A.C. & McCLYMONT, G.L. (1961). Pharmacology and toxicology of carbon tetrachloride in the sheep. 1. Blood levels following ruminal, abomasal and intramuscular administration. *Aust. J. agric. Res.*, **12**, 433–439.

KONDOS, A.C. & McCLYMONT, G.L. *(1965). Indirect anthelmintic action of carbon tetrachloride against Fasciola hepatica. Nature, Lond.*, **206**, 846–847.

KURIYAMA, H., OSA, T. & TOIDA, N. (1966). Effect of tetrodotoxin on smooth muscle cells of the guinea-pig taenia coli. *Br. J. Pharmac. Chemother.*, **27**, 366–376.

SEWELL, M.M.H., HAMMOND, J.A. & DINNING, D.C. (1968). Studies on the aetiology of anaemia in chronic fascioliasis in sheep. *Br. vet. J.*, **124**, 160–168.

STEPHENSON, W. (1947). Physiological and histochemical observations on the adult liver fluke, *Fasciola hepatica* L. 1. Survival *in vitro. Parasitology*, **38**, 116–122.

STREET, H.V. (1969). The use of gas-liquid chromatography in clinical chemistry. In *Advances in Clinical Chemistry*, vol. **12**, ed. Bodansky, O. & Stewart, C.P., pp. 217–307. New York and London: Academic Press.

THORPE, E. (1967). A histochemical study with *Fasciola hepatica. Research in Veterinary Science*, **8**, 27–36.

*(Received April 28, 1976.*
*Revised May 28, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 401–406

# THE EFFECT OF NANDROLONE, AN ANABOLIC STEROID ON PUTRESCINE METABOLISM IN THE MOUSE

S. HENNINGSSON & ELSA ROSENGREN
Institute of Physiology, University of Lund, S–223 62 Lund, Sweden

1 The catabolism of injected $^{14}$C-putrescine was studied in mice treated with nandrolone phenpropionate, an anabolic steroid.

2 The putrescine was rapidly metabolized; almost 50% of the injected radioactivity was recovered within 2 h as $^{14}CO_2$ in the expired air.

3 Considerable amounts of radioactive γ-aminobutyric acid (GABA) and an unidentified compound were found in the kidney and in the urine in addition to radioactive putrescine, spermidine and spermine both in controls and nandrolone-treated mice.

4 Nandrolone elevated the concentration of endogenous putrescine in the kidney and urine, eightfold and twentyfold, respectively, and the concentrations of spermidine and spermine were also increased.

5 After the injection of $^{14}$C-putrescine the incorporation of $^{14}$C into spermidine was significantly increased in the kidney of mice receiving nandrolone.

## Introduction

Evidence is accumulating to show that putrescine and its metabolites spermidine and spermine are ubiquitously present in animal tissues. It is known that putrescine can be metabolized to spermidine and spermine and it has been proposed that putrescine formation is the rate-limiting step in the synthesis of these polyamines (Jänne & Raina, 1968; Williams-Ashman, Pegg & Lockwood, 1969). Relatively little is known about the oxidation of putrescine in animal tissues (Raina & Jänne, 1975). Seiler and his coworkers were the first to show that [$^{14}$C]-putrescine could be incorporated into γ-aminobutyric acid (GABA) in mammalian tissues (Seiler, Wiechmann, Fischer & Werner, 1971; Seiler & Al-Therib, 1974). GABA was first shown to be a constituent of non-neural tissue in the late sixties (Zachmann, Tocci & Nyhan, 1966; Whelan, Schriver & Mohyuddin, 1969). However, the kidney is the only non-neuronal organ in which its concentration is considerable (Lancaster, Mohyuddin, Schriver & Whelan, 1973). GABA is alleged to stimulate protein synthesis (Baxter, 1970). This is of particular interest since it has been suggested that polyamines might be implicated in growth processes and in RNA metabolism (see e.g. Cohen, 1971; Russell, 1973; Raina & Jänne, 1975). Thus GABA formed from putrescine may have a similar regulatory function in growth processes.

A distinct sex difference in putrescine formation has been disclosed (Henningsson & Rosengren, 1975). Putrescine formation by mouse kidney was low in both sexes up to 3 weeks of age. Thereafter the amine formation in the male increased conspicuously, whereas that of the female kidney remained low. Testosterone administration resulted in an elevated biosynthesis of putrescine in the mouse kidney and caused a striking increase in the urinary excretion of free putrescine (Grahn, Henningsson, Kahlson & Rosengren, 1973). Nandrolone, an anabolic steroid with low androgenic activity produced a hundredfold elevation of putrescine formation (to be published).

This significantly increased formation of putrescine in the mouse treated with nandrolone led us to investigate the metabolic fate of [$^{14}$C]-putrescine in mice. Special attention was paid to the kidney as its growth is stimulated by this anabolic steroid. The present paper also describes changes in the concentrations of endogenous putrescine and polyamines in the kidney and urine after administration of nandrolone.

## Methods

Castrated adult mice (30 g, Naval Medical Research Institute strain) were used. They were fed a standard pellet diet and water *ad libitum* except when urine was collected in which case the mice were given 4 g daily of a partly synthetic diet, the composition of which was given in an earlier paper (Kahlson, Rosengren & Westling, 1958). The putrescine content of this diet was 2 nmol/g. The mice were kept individually in metabolic cages, which provided facilities for collecting urine and faeces separately. The beaker in which urine was collected contained 10 drops of 5 M HCl.

Nandrolone phenpropionate (the phenylpropionate ester of 19-nortestosterone; Durabolin, Organon) was suspended in arachis oil; controls were given arachis oil only. 1,4[$^{14}$C]-putrescine (New England Nuclear) was dissolved in 0.9% NaCl solution (saline). All injections were given s.c.

### *Preparation of samples for analysis*

Urine samples were prepared as follows: sulphosalicylic acid (30 mg) and ethylenediamine tetraacetic acid (EDTA, 1 mg) were added to each ml of urine and the mixture was kept cold (4 °C) at least 3 h before the pH was adjusted to 2.0–2.5 with NaOH. The solution was then filtered through a filter with a pore size of 0.22 µm (Millipore Corp., Bedford, Mass. 01730). Extracts of homogenized kidney were prepared in a similar way.

### *Determination of putrescine and its metabolites*

The separation and quantitative estimation of the relevant compounds in urine and kidney extracts were carried out at 60°C on a column of Durrum DC 6 A (7.0 × 0.6 cm) using the automatic amino acid analyser, LKB-BIOCAL 3201; the principle of the method has been published (Hatano, Sumizu, Rokushika & Murakami, 1970). In our modification of the method for separation of putrescine, spermidine and spermine four buffers made up of sodium citrate and sodium chloride were used in sequence with a flow rate of 80 ml/hour. The composition and the run time of the buffers were: Buff. A: 0.20 M Na$^+$, pH 3.5, 44 min; Buff. B: 0.35 M Na$^+$, pH 6.3, 44 min; Buff. C: 0.9 M Na$^+$, pH 6.3, 48 min; Buff. D: 2.8 M Na$^+$, pH 5.8, 40 minutes. The amines were quantified by measuring the peak areas of the curve representing the ninhydrin coloured complex.

For analysis of radioactive compounds the same amino acid analyser was used. After the stream had passed the column, it was split according to the method of Lou (1973). One half of the effluent was used for ninhydrin reaction in the usual way and the other half proceeded to a fraction collector by means of which a total of 70 fractions (2.0 ml each) were collected. To 1 ml of these fractions 9 ml of the liquid scintillator Instagel (Packard) was added and the radioactivity was measured in a liquid scintillation spectrometer (Packard TRI-CARB, 2002).

The final identification of the compounds in relevant fractions of eluates from the amino acid analyser was achieved as follows. Putrescine, spermidine and spermine were isolated by electrophoresis and determined as described in an earlier paper (Henningsson & Rosengren, 1975). The identity of GABA was confirmed by rechromatography on the column of the amino acid analyser but in a different buffer system (0.127 M Na$^+$, 0.025 M citrate, pH 2.88) as described by Hamilton (1962) for separation of amino acids. Further, co-chromatography with authentic GABA showed a coincident peak.

### *Determination of expired $^{14}CO_2$*

Mice were injected with [$^{14}$C]-putrescine (2.5 µCi, 50 µg) and immediately placed in a cylindrical plastic chamber. A flow system provided air through the chamber in a constant stream. This air-stream was finally directed to a series of vials containing 5 ml of hyamine 10-X for quantitative absorption of the $^{14}CO_2$ excreted. Aliquots (0.1 ml each) of the hyamine were transferred to counting vessels containing 10 ml of Bray scintillation solution (Bray, 1960) and the radioactivity was determined.

## Results

### *Radioactivity in the kidney and urine after administration of [$^{14}$C]-putrescine*

Castrated mice were injected with nandrolone (0.1 mg daily for 3 days); controls received arachis oil only. A subcutaneous injection of [$^{14}$C]-putrescine was given on the 3rd day and the amount of [$^{14}$C]-compounds in the kidney was determined 1 h and 24 h later and in the urine 24 h later.

The results of the determinations of [$^{14}$C]-compounds found in the kidney are presented in Figure 1 where for the sake of simplicity the radioactivity is given as nCi/g. The combined weight of the two kidneys of castrated mice was about 260 mg and of mice given nandrolone (0.1 mg daily for 3 days) 360 mg. In both groups of mice 40% of the total radioactivity found in the kidneys 1 h after the injection of [$^{14}$C]-putrescine was unchanged [$^{14}$C]-putrescine. After 24 h this portion was reduced to 5% of the total radioactivity.

A major part of the radioactivity found in the mouse kidney after the injection of [$^{14}$C]-putrescine was apparently incorporated into the spermidine skeleton; this was particularly noticeable in determinations made 1 h after the injection. Further, it was found that at 1 h after administration of [$^{14}$C]-putrescine, the concentration of labelled spermidine was twice as high in the kidney of the nandrolone-treated animals as it was in the kidneys of the controls while at 24 h no difference was found between the two groups.

Appreciable amounts of [$^{14}$C]-spermine were found in both groups of mice 1 h and 24 h after the injection of [$^{14}$C]-putrescine. The highest concentrations were noted at 24 hours. As seen from Figure 1, the

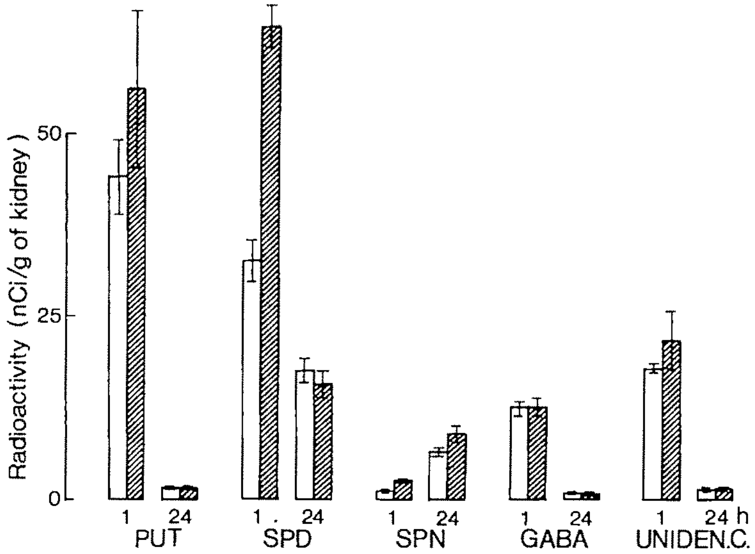


Figure 1 Radioactivity (nCi/g) in the kidney 1 h and 24 h after [$^{14}$C]-putrescine (2.5 μCi s.c.). Open columns: controls; hatched columns: mice injected with nandrolone (0.1 mg daily for 3 days). PUT=putrescine, SPD=spermidine, SPN=spermine, GABA=γ-aminobutyric acid, UNIDEN. C.=unidentified compound. Each column is the mean ± s.e. mean of 4 observations. Total kidney weights: controls 260 mg, nandrolone-treated animals 360 mg; no significant difference in body weight.

spermidine concentrations were reduced and those of spermine elevated 24 h after the injection; this observation may be due to the fact that the labelled carbon of putrescine is incorporated into spermidine before it can be transferred to the spermine molecule.

In the mouse kidney considerable amounts of radioactive carbon were recovered as GABA following the subcutaneous injection of [$^{14}$C]-putrescine (Figure 1). In addition to the above mentioned radioactive compounds found in the kidney extract after injection of [$^{14}$C]-putrescine, an unidentified radioactive compound was eluted from the column. This substance appeared before GABA in the eluate and was distinctly separated from the latter. The amount of this unknown compound was greater than that of GABA and it appeared that the concentration of the unidentified compound as well as that of GABA was several times higher at 1 h after putrescine administration than at 24 hours. No quantitative differences were seen in the amounts of GABA or the unidentified compound between the kidneys of animals treated with nandrolone or the controls.

From the mice in which [$^{14}$C]-putrescine metabolites were determined in the kidneys 24 h after [$^{14}$C] were incorporated into GABA. An unidentified examined its [$^{14}$C]-compounds. Of the total radioactivity injected 20% appeared in the urine in the untreated controls and 39% in the group of mice given nandrolone. Table 1 shows that about two-thirds of the total urinary radioactivity was excreted as unmetabolized putrescine. Significant amounts of the injected [$^{14}$C] was incorporated into GABA. An unidentified carbon-labelled compound was also present in the urine and appeared in the same fraction of the column

Table 1 Radioactivity (nCi/24 h) in urine after injection of [$^{14}$C]-putrescine in 6 controls and 6 mice injected with nandrolone (0.1 mg daily for 3 days). Means ± s.e. means and the degrees of significance between nandrolone-treated animals and controls are given. For abbreviations see Figure 1.

| | *Total radioactivity* | *Putrescine* | *Spermidine* | *Spermine* | *GABA* | *Unidentified compound* |
|---|---|---|---|---|---|---|
| Controls | 490.8 ± 118.05 | 300.5 ± 94.33 | 2.5 ± 0.38 | 0.4 ± 0.12 | 17.2 ± 2.65 | 128.2 ± 13.11 |
| Nandrolone | 975.4 ± 167.75* | 694.9 ± 126.70* | 8.0 ± 1.00** | 0.8 ± 0.20 | 21.8 ± 0.90 | 178.4 ± 22.23 |

*$P<0.05$, **$P<0.01$.

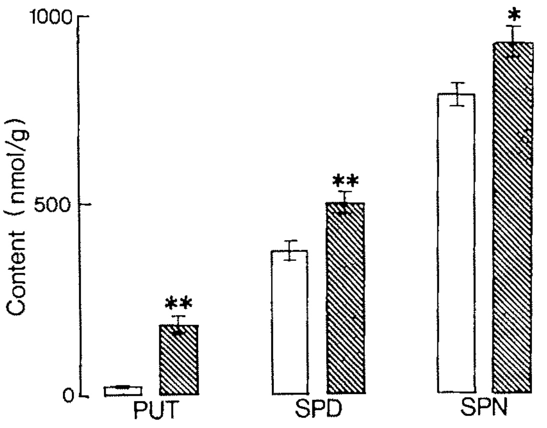


**Figure 2** Concentrations of endogenous putrescine (PUT), spermidine (SPD) and spermine (SPN) in the kidneys in controls (open columns) and in mice injected with nandrolone (0.1 mg daily for 3 days; hatched columns). The content is expressed in nmol/g; each column is the mean ± s.e. mean of 8 observations. The degree of significance between nandrolone-treated animals and controls is given. $^*P<0.02$, $^{**}P<0.01$.

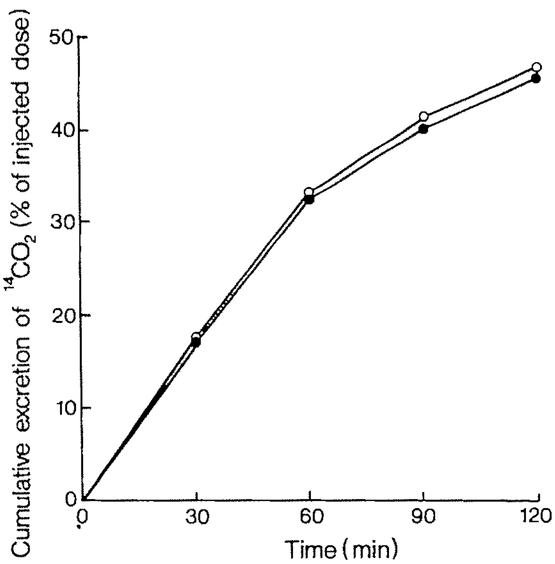


**Figure 3** Cumulative $^{14}CO_2$ excretion following injection of [$^{14}$C]-putrescine in mice injected with nandrolone (0.1 mg daily for 3 days; ●) and in controls (○). Mean values of 3 experiments.

eluate in which we had observed the unidentified radioactive substance in the kidney extracts. The amount of this substance in the urine was almost 10 times that of GABA. Less radioactivity was found in the urinary polyamines spermidine and spermine. The excretion of [$^{14}$C]-spermidine was significantly higher in mice treated with nandrolone.

### *Content of endogenous putrescine, spermidine and spermine in the kidney and urine after nandrolone administration*

The content of endogenous putrescine, spermidine and spermine in mouse kidney and urine after nandrolone injections was determined and compared with that of castrated control mice. The analysis was done on the same mice which were used in the experiments with [$^{14}$C]-putrescine (see above). In the kidney the content of each of the amines determined was elevated after nandrolone administration, particularly that of putrescine which was increased eight times (Figure 2). Still greater differences were found between controls and nandrolone-injected animals in the urinary excretion; the nandrolone-treated group excreted 20 times more putrescine and 5 times more spermidine than the controls (Table 2). The excretion of spermine was low and not significantly different in the two groups.

### *Radiorespirometric recording of $^{14}CO_2$ after injection of labelled putrescine*

Figure 3 shows the time course of the occurrence of $^{14}CO_2$ in the expired air during the 2 h period

**Table 2** Urinary excretion (nmol/24 h) of putrescine, spermidine and spermine in 6 controls and 6 mice injected with nandrolone (0.1 mg daily for 3 days). Means ± s.e. means and the degrees of significance between nandrolone-treated animals and controls are given. The volumes of the 24 h urinary samples were 4 to 5 ml in both groups.

| | *Putrescine* | *Spermidine* | *Spermine* |
|---|---|---|---|
| Controls | 198.4 ± 34.02 | 29.3 ± 3.96 | 2.2 ± 1.40 |
| Nandrolone | 4307.9 ± 457.60* | 137.1 ± 26.64* | 4.6 ± 2.48 |

$^*P<0.01$.

following the injection of [$^{14}$C]-putrescine (2.5 µCi). Nearly 50% of the injected dose was expired as $^{14}CO_2$ within 2 hours. No difference was seen between the control group and the group treated with nandrolone. In mice pretreated with the diamine oxidase inhibitor aminoguanidine (5 mg/kg) half an hour before the [$^{14}$C]-putrescine injection the production of $^{14}CO_2$ was lowered to 3% of the injected radioactivity.

## Discussion

A rather conspicuous finding in this study was the presence of radioactive GABA in the kidney and urine after injection of [$^{14}$C]-putrescine. The incorporation of putrescine carbon into GABA in rat liver and brain has been reported (Seiler *et al.*, 1971). During the preparation of this report Seiler and his group reported also the urinary excretion of radioactive GABA after [$^{14}$C]-putrescine injection in mice but only after pretreatment with an inhibitor of γ-aminobutyrate aminotransferase, an enzyme that hastens the degradation of GABA (Seiler & Eichentopf, 1975). The discrepancy between Seiler's and our results cannot yet be explained. It may be due to differences in the methods used to assay GABA. We have found the automatic amino acid analyser very useful for determinations of amines and, when combined with the split-stream technique as in this study, also for determinations of radioactivity in various amine metabolites, such as GABA.

Jänne (1967) showed in rats that one-third of the injected dose of [$^{14}$C]-putrescine was expired as $^{14}CO_2$ within 2 h, an observation which we have confirmed in mice. [$^{14}$C]-GABA administered to rats is rapidly degraded to $^{14}CO_2$ via the Krebs' tricarboxylic acid cycle (Wilson, Hill & Koeppe, 1959). The present results, together with the reports of Seiler's group (Seiler *et al.*, 1971; Seiler & Eichentopf, 1975), show that GABA is an intermediate in the catabolism of putrescine to carbon dioxide.

One hour after injection of putrescine, [$^{14}$C]-spermidine was present in the kidney in amounts similar to that of the parent compound. Further, in mice treated with nandrolone [$^{14}$C]-spermidine was elevated at 1 h and the radioactive spermidine disappeared faster, indicating an accelerated turnover rate. These observations appear useful in attempts to explore possible functions of the amine.

As mentioned in the Introduction recent studies on rapidly growing cells and tissues suggest a connection between synthesis of polyamines on the one hand and nucleic acid and protein metabolism on the other. We report an increased accumulation of endogenous putrescine and polyamines in the kidney under the influence of an anabolic agent which is reflected in the urinary excretion. Besides, the urinary excretion of radioactive carbon incorporated into spermidine was elevated indicating an increased formation of this amine. The elevation of the total urinary radioactivity owing to an increased [$^{14}$C]-putrescine excretion in the anabolic steroid treated mice may be explained by a heightened renal blood flow which is reported to occur in mice treated with testosterone propionate (Broulik, Kochakian & Dubovsky, 1969).

Evidence is accumulating that in patients with tumours and leukaemia the excretion of polyamines is elevated; determination of the rate of polyamine excretion has been suggested as a test in the early detection of cancer (Russell, Levy, Schimpff & Hawk, 1971). The present study showed that more urinary radioactive carbon was found in the fractions of GABA and in the unidentified compound than in the polyamine fractions pointing out the relative importance of the different pathways in putrescine metabolism. If it were possible to determine endogenous amounts of the as yet unidentified putrescine metabolite the estimation of this compound in the urine may be a more sensitive tool in revealing early cancer.

This work was supported by grants from the Swedish Medical Research Council B 76-04X-02212-09B and from the Medical Faculty, University of Lund.

## References

BAXTER, C.F. (1970). The nature of γ-aminobutyric acid. In *Handbook of Neurochemistry*, Vol. 3. ed. Lajtha, A., pp. 289–353. New York—London: Plenum Press.

BRAY, G. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analyt. Biochem.*, **1**, 279–285.

BROULIK, P., KOCHAKIAN, C.D. & DUBOVSKY, J. (1969). Influence of testosterone propionate on renal blood flow in mice. *Fedn Proc.*, **28**, 715.

COHEN, S.S. (1971). *Introduction to the Polyamines*. New Jersey: Prentice-Hall.

GRAHN, B., HENNINGSSON, S., KAHLSON, G. & ROSENGREN, E. (1973). Alterations in the activities of ornithine and histidine decarboxylases in mice. *Br. J. Pharmac.*, **48**, 113–120.

HAMILTON, P.B. (1962). Ion exchange chromatography of amino acids—microdetermination of free amino acids in serum. *Ann. N.Y. Acad. Sci.*, **102**, 55–75.

HATANO, H., SUMIZU, K., ROKUSHIKA, S. & MURAKAMI, F. (1970). Automatic liquid chromatography of primary mono- and diamines on a cation-exchange resin. *Analyt. Biochem.*, **35**, 377–383.

HENNINGSSON, S. & ROSENGREN, E. (1975). Biosynthesis of histamine and putrescine in mice during post-natal development and its hormone dependence. *J. Physiol., Lond.*, **245**, 467–479.

JÄNNE, J. (1967). Studies on the biosynthetic pathway of polyamines in rat liver. *Acta physiol. scand., Suppl.* 300, 1–71.

JÄNNE, J. & RAINA, A. (1968). Stimulation of spermidine synthesis in the regenerating rat liver: Relation to increased ornithine decarboxylase activity. *Acta chem. scand.*, **22**, 1349–1351.

KAHLSON, G., ROSENGREN, E. & WESTLING, H. (1958). Increased formation of histamine in the pregnant rat. *J. Physiol., Lond.*, **143**, 91–103.

LANCASTER, G., MOHYUDDIN, F., SCHRIVER, C.R. & WHELAN, D.T. (1973). A γ-aminobutyrate pathway in mammalian kidney cortex. *Biochim. Biophys. Acta*, **297**, 229–240.

LOU, M.F. (1973). A split stream ion exchange chromatographic method for isolating amino acids and peptides. *Analyt. Biochem.*, **55**, 51–56.

RAINA, A. & JÄNNE, J. (1975). Physiology of the natural polyamines putrescine, spermidine and spermine. *Med. Biol.*, **53**, 121–147.

RUSSELL, D.H. (1973). In *Polyamines in Normal and Neoplastic Growth*. New York: Raven Press.

RUSSELL, D.H., LEVY, C.C., SCHIMPFF, S.C. & HAWK, I.A. (1971). Urinary polyamines in cancer patients. *Cancer Res.*, **31**, 1555–1558.

SEILER, N. & AL-THERIB, M.J. (1974). Putrescine catabolism in mammalian brain. *Biochem. J.*, **144**, 29–35.

SEILER, N. & EICHENTOPF, B. (1975). 4-Aminobutyrate in mammalian putrescine catabolism. *Biochem. J.*, **152**, 201–210.

SEILER, N., WIECHMANN, M., FISCHER, H.A. & WERNER, G. (1971). The incorporation of putrescine carbon into γ-aminobutyric acid in rat liver and brain *in vivo*. *Brain Res.*, **28**, 317–325.

WHELAN, D.T., SCHRIVER, C.R. & MOHYUDDIN, F. (1969). Glutamic acid decarboxylase and gamma-aminobutyric acid in mammalian kidney. *Nature, Lond.*, **224**, 916–917.

WILLIAMS-ASHMAN, H.G., PEGG, A.E. & LOCKWOOD, D.H. (1969). Mechanisms and regulation of polyamine and putrescine biosynthesis in male genital glands and other tissues of mammals. In *Advances in Enzyme Regulation*, Vol. 7, ed. Weber, G., pp. 291–323. New York: Pergamon Press.

WILSON, W.E., HILL, R.J. & KOEPPE, R.E. (1959). The metabolism of γ-aminobutyric acid-4-$C^{14}$ by intact rats. *J. biol. Chem.*, **234**, 347–349.

ZACHMANN, M., TOCCI, P. & NYHAN, W.L. (1966). The occurrence of γ-aminobutyric acid in human tissues other than brain. *J. biol. Chem.*, **241**, 1355–1358.

*(Received May 5, 1976.*
*Revised June 22, 1976)*

PROCEEDINGS OF THE

# British and French Pharmacological Societies

15th–17th September, 1976

THE UNIVERSITY OF OXFORD

## COMMUNICATIONS

### The effects of dobutamine in the early stages of acute experimental myocardial infarction in the dog

R.J. MARSHALL & J.R. PARRATT

*Department of Physiology and Pharmacology, University of Strathclyde and Department of Surgery, University of Glasgow*

It has been reported (Tuttle, Pollock, Todd & Tust, 1973) that the $\beta$-adrenoceptor stimulant, dobutamine, reduces the area of infarction which results from coronary artery occlusion in dogs. The reasons for this interesting action are uncertain but could be related to changes in blood flow and oxygen availability. We have now investigated the effects of dobutamine on these parameters, using methods previously described (Marshall & Parratt, 1976), simultaneously in both normal and acutely ischaemic regions of the left ventricular wall, 1 – 2 h after acute coronary occlusion in anaesthetized greyhounds. These effects have been related to changes in the extent, and degree, of ischaemic injury as assessed by epicardial S-T segment mapping.

The results (Table 1) demonstrate the positive inotropic effect of dobutamine in the early stages of infarction. In contrast to other cardiac stimulants (Ledingham, Marshall & Parratt, 1973), this was achieved without a significant increase in myocardial oxygen consumption. Similar effects were observed with a dose of 20 $\mu g\, kg^{-1}\, min^{-1}$ except that the increase in heart rate (189 ± 7 to 219 ± 8 beats/min) was more pronounced. Blood flow in the ischaemic myocardium was consistently increased by dobutamine and oxygen extraction decreased. The

**Table 1** The effects of dobutamine (10 μg $\text{kg}^{-1}\,\text{min}^{-1}$) following acute coronary artery occlusion in anaesthetized greyhounds

| | *Pre-dobutamine* | *Post-dobutamine* |
|---|---|---|
| Mean systemic blood pressure (mmHg) | 133 ± 3 | 134 ± 3 |
| Heart rate (beats/min) | 188 ± 13 | 210 ± 10* |
| LV dP/dt max (mmHg/s) | 2278 ± 225 | 3313 ± 297* |
| LVEDP (mmHg) | 7.7 ± 1.3 | 5.5 ± 1.2* |
| Cardiac output (l/min) | 2.2 ± 0.4 | 2.6 ± 0.3* |
| Transventricular driving pressure (mmHg) | 3.5 ± 2.8 | 8.2 ± 3.2* |
| Myocardial blood flow (normal region (ml/min) | 75 ± 12 | 96 ± 14* |
| Coronary sinus oxygen content (ml/100 ml) | 9.7 ± 0.5 | 12.4 ± 0.3* |
| Myocardial blood flow (ischaemic region) (ml 100 $g^{-1}\,\text{min}^{-1}$) | 27 ± 6 | 34 ± 6* |
| Coronary vein oxygen content (ml/100 ml) | 8.5 ± 0.1 | 13.1 ± 1.3* |

* $P < 0.05$ Paired *t* test.

dobutamine-induced increases in myocardial contractility and work did not extend the ischaemic area since epicardial S-T segment elevation was slightly reduced or remained unchanged. In contrast, isoprenaline in a dose (0.1 μg $\text{kg}^{-1}\,\text{min}^{-1}$) that increased dP/dt max. to the same degree, consistently *increased* S-T segment elevation, an effect related to the more pronounced tachycardia. Dobutamine and isoprenaline also differed in their effects on local myocardial temperatures, dobutamine causing significantly smaller increases especially in the subendocardial layers of the infarcting zone.

These effects of dobutamine on blood flow, temperature and S-T elevation may result from an increase in transventricular pressure (Marshall & Parratt, 1974) and thus favour subendocardial blood flow.

### References

LEDINGHAM, I. McA., MARSHALL, R.J. & PARRATT, J.R. (1973). Drug-induced changes in blood flow in normal and ischaemic regions of the canine myocardium. *Br. J. Pharmac.*, **47**, 626–627P.

MARSHALL, R.J. & PARRATT, J.R. (1974). Drug-induced changes in blood flow in the acutely ischaemic canine myocardium; relationship to subendocardial driving pressure. *Clin. exp. Pharm. Physiol.*, **1**, 99–112.

MARSHALL, R.J. & PARRATT, J.R. (1976). Comparative effects of propranolol and practolol in the early stages of experimental canine myocardial infarction. *Br. J. Pharmac.*, **57**, in press.

TUTTLE, R.R., POLLOCK, G.D., TODD, G. & TUST, R. (1973). Dobutamine: containment of myocardial infarction size by a new inotropic agent. *Circulation*, **48**, Suppl. IV, IV–132.

---

## Comparative effects of chloralose and pentobarbital on atrioventricular conduction in the dog

R. DELAIGUE, P. DUCHENE-MARULLAZ, G. GUEORGUIEV & J.P. KANTELIP

*Department of Pharmacology, Faculty of Medicine, 63001 Clermont-Ferrand Cedex, France*

Two electrodes were implanted in mongrel dogs of either sex: one at the upper end of the atrioventricular node, the other 2 cm away in the atrial myocardium. The electrode leads emerged in the region of the back of the neck. It was then possible to study in the conscious dog atrioventricular conduction by stimulating the atria at increasing frequencies in successive stages. In the unanaesthetized animal at rest, there was a difference in the sensitivity to the atrial stimulation depending on the stage of the respiratory cycle: minimal (135 ± 15 beats/min) during expiratory bradycardia, maximal (240 ± 14 beats/min) during inspiration.

Chloralose (0.8 g/kg i.v.) did not substantially modify sinus rate or atrioventricular conduction ($n = 7$). It did not alter these two stages but decreased the positive chronotropic effects and to a lesser extent, the dromotropic effects of atropine (0.1 mg/kg i.v.).

Pentobarbital (25 mg/kg i.v.) suppressed respiratory arrhythmia and its phasic influence on conduction. It decreased both the positive chronotropic and dromotropic influence of atropine.

In conclusion, pentobarbital is contraindicated as an anaesthetic for the study of atrioventricular conduction in the dog. Although chloralose seems preferable, it also attenuates if to a lesser extent, the facilitating dromotropic influence of atropine.

---

## Heterogeneous reactivity of atrial and ventricular cardiac contractile tissue

J.P. FARGES, G. FAUCON & M. OLLAGNIER

*Department of Medical Pharmacology, University Claude Bernard, Lyons, France*

In order to investigate induced changes in the effective refractory period of the atrial and ventricular cardiac muscle by means of cardiotropic drugs, the interventricular septum was removed in 24 mongrel dogs under chloralose anaesthesia and during total cardio-pulmonary by-pass to obtain independent atrial and ventricular contractions. The comparison of the variations of the effective refractory period in the right atrium and in one of the ventricles was checked by the extra-stimulus method, the contractile fibres being directly under the dependence of an electric pacer (frequency: 150/min) to prevent rhythm-induced changes of the refractory period.

Acetylcholine (0.5, 1 and 2 mg $kg^{-1}$ $min^{-1}$) considerably shortened the effective refractory period of atrial cardiac fibres ($P < 0.05$), but did not provoke any change in the ventricular contractile tissue.

Ouabain (30, 40 and 50 µg/kg) and quinidine (5, 10 and 20 mg/kg) induced larger variations in the refractory period of atrial contractile tissue than in the ventricular tissue.

Nevertheless, the heterogeneous reactivity of the contractile tissue, present both in the atrium and in the ventricle, is not a general law. As a matter of fact, the isoproterenol (0.5, 1 and 2 µg/kg/min)-induced shortening of the effective refractory period for both atrial and ventricular tissue is not significantly different.

---

## A method for experimental study of arrhythmias following myocardial infarction in closed-chest dog. Its use as a test for antiarrhythmic drugs

B. DUPUIS, P. GAUTIER, G. RICHER & M. SERRE

*Centre de Recherches Clin-Midy—F 34 Montpellier*

A small metallic coil was wedged distally either in the anterior interventricular artery or in the circumflex artery through coronary catheterization under X-ray control. While the animal recovered from anaesthesia a thrombosis developed progressively. The ECG was monitored by telemetry on a 24 h basis. All changes of the cardiac rhythm and/or of the QRS complexes were analysed on a specially built electronic device.

The method has been applied to 50 dogs on which a total of 55 infarctions were elicited. All animals but one showed ventricular arrhythmias. Two of them died from ventricular fibrillation. More than half of the dogs had episodes of ventricular tachycardia. In all cases trains of ventricular extrasystoles were observed. The delay in onset of the arrhythmia was variable, ranging from 70 min to 30 hours. The duration of the periods of arrhythmia ranged from one to six days.

Ajmaline, amiodarone, phenytoin, disopyramide, quinidine, lidocaine, procainamide, propranolol and verapamil were administered during various types of ventricular arrhythmias.

## Effects of tryptamine on mechanical and electrical activities of sino-atrial frog auricular trabeculae

D. GARNIER, N. GOUPIL, J. NARGEOT, B. POURRIAS & O. ROUGIER

*Department of Pharmacology, Delalande Centre of Research, 10 rue des Carrières 92500 Rueil-Malmaison and Laboratories of Physiology, Facultés des Sciences de Tours et de Lyon*

The effects of tryptamine on ionic currents were studied in frog atrial fibres by means of a double sucrose gap technique associated with a transducer RCA 5734 for the recording of mechanical tension.

Under current clamp conditions, tryptamine ($5 \times 10^{-6}$ g/ml), without modifying the membrane resistance, caused a significant increase in the slow phase of depolarization and an increase in plateau duration. The rate of rise of the action potential was not affected, but tryptamine produced a marked increase in contraction strength. When rapid inward current was blocked by tetrodotoxin and external calcium was removed, the inotropic effect was still observed. In sodium-free medium, it disappeared.

Under voltage clamp conditions, calcium inward current and the phasic component of the contraction were not modified. In calcium-free medium, the increase in contraction amplitude may be related to the important rise of slow sodium inward current. Unlike the description by Horackova & Vassort (1974), our observations show that variation of the contraction strength responds, beat to beat, to the variation of slow inwatd sodium current.

These results suggest that the inotropic property of tryptamine is mainly due to an increase of the slow inward current and that sodium induces calcium release.

### Reference

HORACKOVA, M. & VASSORT, G. (1974). Excitation contraction coupling in frog heart—Effect of Veratrine. *Pflügers Archiv. für Physiol.*, **352**, 292–302.

---

## Autonomous control of the heart, myocardial blood flow and S-T segment in canine ischaemic myocardium

A. BERDEAUX, J.R. BOISSIER & J.F. GIUDICELLI

*Laboratoire de Pharmacologie II, 15 rue de l'Ecole-de-Médecine 75270—Paris, Cedex 6, France*

The effects of cardiac parasympathetic stimulation and of bilateral stellectomy on regional myocardial blood flow (measured by tracer microspheres) and the S-T segment were investigated in normal and ischaemic regions on a reproducible model of temporary coronary occlusion in the dog.

The control coronary occlusion did not modify heart rate but induced in the ischaemic area an increase in S-T segment and a decrease in endo- (−71%) and epicardial (−49%) blood flows, resulting in a diminished endo/epi ratio (ranging from 0.40 ± 0.04 to 0.56 ± 0.08 as compared with 0.97 ± 0.02 to 1.04 ± 0.04 in the non-ischaemic region).

Bilateral stellectomy decreased heart rate (−33%), lowered S-T segment elevation (−62%) and further reduced endo- (−35%) and epicardial (−30%) blood flows in the ischaemic area without inducing redistribution (endo/epi ratio = 0.52 ± 0.05 as compared with 0.56 ± 0.08). Endo- and epicardial blood flows were also reduced (−40%) in non-ischaemic areas and endo/epi ratio was not modified.

Vagal stimulation (30 Hz, 2 ms, 3 V) decreased heart rate (−16%) and S-T segment elevation (−30%) and increased slightly but non-significantly endo- and epicardial blood flows (+11%) and endo/epi ratio (+5%) in ischaemic areas. However, at 70 Hz, vagal stimulation still reduced heart rate (−28%) and S-T segment elevation (−44%) but significantly increased endo (+108%) and epicardial (+92%) blood flows resulting in an endo/epicardial blood flow redistribution (endo/epi ratio: 0.56 ± 0.03 as compared with 0.40 ± 0.04, $P < 0.01$). These effects of vagal stimulation at high frequencies were maintained under electrosystolic pacing of the heart but abolished under combined electrosystolic pacing and atropine administration.

It is concluded that (1) there is no correlation between regional blood flows and endo/epi ratio evolution in ischaemic areas on the one hand and bradycardia on the other hand, although there is correlation between decrease in heart rate and lowering of S-T segment, (2) the redistribution phenomenon observed after vagal stimulation at high frequencies is probably related to a muscarinic effect of acetylcholine.

# Atenolol, regional myocardial blood flow and S-T segment in canine ischaemic myocardium

A. BERDEAUX, J.R. BOISSIER &
J.F. GIUDICELLI

*Laboratoire de Pharmacologie II, 15 rue de l'Ecole-de-Médecine 75270—Paris, Cedex 6, France*

The effects of atenolol on regional myocardial blood flow (measured by tracer microspheres) and the S-T segment were compared in normal and ischaemic regions on a reproducible model of temporary coronary occlusion in the dog.

The control coronary occlusion did not modify heart rate but induced in the ischaemic area an increase in S-T segment and a decrease in endo- (−71%) and epicardial (−49%) blood flows, resulting in a diminished endo/epi ratio (0.55 ± 0.05 as compared with 1.02 ± 0.05 in the non-ischaemic region).

Atenolol (1 mg/kg, i.v.) decreased heart rate (−28%), lowered S-T segment elevation (−60%) and further reduced endo- and epicardial blood flows without inducing redistribution (endo/epi ratio: 0.61 ± 0.09 in the ischaemic area). In the non-ischaemic area, atenolol also decreased endo- and epicardial blood flows (−40%) without affecting endo/epi ratio (1.08 ± 0.07). Bilateral stellectomy induced similar effects to atenolol administration.

After bilateral stellectomy, atenolol (1 mg/kg, i.v.) induced no additional effects. Finally, under electrosystolic pacing, atenolol (1 mg/kg, i.v.) no longer lowered S-T segment elevation and did not further modify regional blood flows and endo/epi ratio.

It is concluded that atenolol (1) reduces regional myocardial blood flows and S-T segment elevations, these two phenomena being correlated with the decrease in heart rate, (2) does not induce endo-epicardial blood flow redistribution neither in ischaemic nor in non-ischaemic regions.

---

# An indolizine with an amiodarone-like haemodynamic profile

R.H. CHARLIER, J.A. RAUTHIER &
J.C. RICHARD

*Department of Pharmacological Research, LABAZ Centre Research, 1120—Bruxelles, Belgium*

L 9394 or 2-ethyl-3-(4-γ-di-n-butylaminopropoxy-benzoyl)-indolizine hydrochloride (each 10 mg/kg, 5% aqueous solution) produces amiodarone-like haemodynamic effects when injected i.v. (2 min) into the atropinized (1 mg/kg i.v.) and anaesthetized (sodium pentobarbitone, 30 mg/kg i.v.) mongrel dog. Maximal changes in all measured haemodynamic parameters occurred at the end of the injection-time, except for heart rate for which the maximal change was noted after 5 minutes. The average changes were as follows in 11 treated dogs compared to 18 control dogs receiving saline. Heart rate decreased consistently by 31%. Mean blood pressure fell by 48%, diastolic pressure being more reduced than systolic pressure. Myocardial oxygen consumption computed according to the index of Robinson (1967) diminished sharply by 58%. Cardiac output increased by 74%, stroke volume by 160%. L 9394 also enhanced coronary arterial blood flow (as measured electromagnetically in 10 open chest dogs) by 123% on the average. All these changes were very highly significant ($P < 0.001$). Heart rate decrease lasted for at least 3 h without any change, but the other parameters wore off within different times; 1 h in the case of blood pressure and cardiac output, 20 min in the case of coronary blood flow, while myocardial oxygen consumption was still reduced by 30% ($P < 0.05$) after 1 h and stroke volume was still increased by 30% ($P < 0.05$) at the same time. L 9394 is furthermore endowed with non-competitive adrenoceptor-blocking properties since adrenaline and noradranaline-induced hypertension as well as adrenaline- and isoprenaline-induced tachycardia were markedly antagonized but never blocked.

The overall haemodynamic properties of L 9394 are qualitatively similar to those of amiodarone (Charlier, Deltour, Baudine & Chaillet, 1968), which is a very effective medication for the long term treatment of angina pectoris (Charlier, 1971). Furthermore drug-induced bradycardia has been shown to be a highly desirable property for an antianginal drug (Gomoll & Braunwald, 1973; Charlier, 1974), because heart rate is a major determinant of myocardial oxygen consumption (Braunwald, 1971). It is therefore considered that L 9394 is worthy of a clinical trial in patients with angina.

## References

BRAUNWALD, E. (1971). Control of myocardial oxygen consumption: physiologic and clinical considerations. *Amer. J. Cardiol.*, **27**, 416–432.

CHARLIER, R. (1971). Antianginal Drugs, *Handb. Exp. Pharmacol.*, Springer Verlag, Berlin-Heidelberg-New York, **31**, 255–288.

CHARLIER, R. (1974). Physiopathologie de l'angor et médication antiangineuse à long terme. Conceptions actuelles. *Nouv. Presse Méd.*, **37**, 2407–2410.

CHARLIER, R., DELTOUR, G., BAUDINE, A. & CHAILLET, F. (1968). Pharmacology of amiodarone, an anti-anginal drug with a new biological profile. *Arzn. Forsch.*, **18**, 1408–1417.

GOMOLL, A.W. & BRAUNWALD, E. (1973). Comparative effects of sotalol and propranolol on myocardial contractility. *Arch. int. Pharmacodyn.*, **205**, 338–354.

ROBINSON, B.F. (1967). Relation of heart rate and systolic blood pressure to the onset of pain in angina pectoris. *Circulation*, **35**, 1073–1083.

---

## Oxprenolol in angina pectoris

S.H. TAYLOR & U. THADANI

*University Departments of Cardiovascular Studies and Medicine, The General Infirmary, Leeds*

Symptomatic, ECG and circulatory effects of the $\beta$-adrenoceptor antagonist oxprenolol were evaluated during treadmill walking in a placebo controlled double-blind study in 35 male patients with stable, uncomplicated angiographically-defined angina pectoris. After a single oral dose of oxprenolol (160 mg), plasma concentration, increase in treadmill walking time, reduction in ECG ST depression and attenuation of exercise tachycardia and systolic pressure increase peaked at 1–2 h and thereafter slowly declined over 8 hours.

Plasma concentration at 1 h was linearly related to the oral dose of oxprenolol. Dose-response measurements demonstrated a significant correlation between reduction in anginal pain, ECG ST depression, exercise tachycardia and systolic pressor response and the logarithm of the dose of drug.

In eight patients there was no increase in exercise tolerance, despite similar plasma concentrations and similar ECG and circulatory changes to those in patients with significant extension of angina time.

Oxprenolol, 160 mg twice daily, affords a rapid and effective treatment in the majority of patients with exercise-induced angina pectoris.

---

## Some complementary data on AH 5158, an inhibitor of both $\alpha$- and $\beta$-adrenoceptors

P. HARICHAUX & L. HARY

*Laboratoire de Physiologie, Faculté de Médecine, 12, rue Frédéric Petit, 80036 Amiens, Cedex, France*

Inhibitory properties of AH 5158 (5[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]salicylamide) upon both $\alpha$- and $\beta$-adrenoceptors, mainly in heart ($\beta_1$ receptors) and blood vessels ($\alpha$ receptors) have been reported (Farmer, Kennedy & Levy, 1971; Farmer, Kennedy, Levy & Marshall, 1972; Kennedy & Levy, 1975).

Our own results show that: (a) in guinea-pigs (anaesthetized with urethane), AH 5158 (1–3 mg/kg) inhibits the protective $\beta_2$ effects of isoprenaline (20 µg/kg) and salbutamol (30 µg/kg) against the broncho-constrictive effects of 5-hydroxytryptamine, acetylcholine and histamine; (b) in the isolated rabbit duodenum (Tyrode solution), AH 5158 ($5 \times 10^{-6}$–$10^{-5}$ g/ml) inhibits the relaxing effect of $0.5$–$1 \times 10^{-6}$ isoprenaline ($\beta_2$), $0.5$–$1 \times 10^{-6}$ adrenaline ($\alpha$–$\beta_1$) and $10^{-7}$–$10^{-6}$ noradrenaline ($\alpha$); (c) whilst looking for possible metabolic effects, we established a slight intrinsic effect of AH 5158 (5 and 10 mg/kg) on plasma $K^+$ level in the rat (anaesthetized with urethane) and an inhibitory effect (for the same doses and species) on sympathomimetically-induced hyperglycaemia.

These results bring additional data to the pharmacological properties of AH 5158, which might have therapeutic applications.

### References

FARMER, J.B., KENNEDY, I. & LEVY, G.P. (1971). Some characteristics of AH 5158, a drug which blocks both $\alpha$- and $\beta$-adrenoceptors. *J. Pharmac.*, **2**, 192–194.

FARMER, J.B., KENNEDY, I., LEVY, G.P. & MARSHALL, R.J. (1972). Pharmacology of AH 5158; a drug which blocks both $\alpha$- and $\beta$-adrenoceptors. *Br. J. Pharmac.*, **45**, 660–675.

KENNEDY, I. & LEVY, G.P. (1975). Combined $\alpha$- and $\beta$-adrenoceptor blocking drug AH 5158: further studies on $\alpha$-adrenoceptor blockade in anaesthetized animals. *Br. J. Pharmac.*, **53**, 585–592.

# Effect of (±)-propranolol on the recovery of urinary concentration process after frusemide, in the rat

J.L. IMBS, M. SCHMIDT & J. SCHWARTZ

*Institut de Pharmacologie, Faculté de Médecine, 11, rue Humann, 67000 Strasbourg, France*

Frusemide eliminates the intrarenal sodium concentration gradient. When its effect wears off, the recovery of urinary concentration process can be followed. In anaesthetized dogs, propranolol delays this recovery (Imbs, Schmidt, Belhadj-Mostefa & Schwartz, 1975). We have observed the effect of propranolol on the recovery of the sodium cortico-papillary gradient after frusemide, in conscious male Wistar rats. (a) Propranolol does not modify the diuretic action of frusemide but keeps urinary osmolality down: $579 \pm 40$ (s.e. mean, $n=20$) instead of $901 \pm 78$ ($P < 0.001$), and $1236 \pm 105$ instead of $1671 \pm 60$ m osmol/kg ($P < 0.005$) respectively 4 h and 8 h after frusemide injection. (b) Papillary and cortical sodium concentrations were measured according to Atherton, Hai & Thomas (1968), 30 min, 1, 2, or 3 h after injecting 4 groups of 20 rats with frusemide (20 mg/kg). Half these rats had been treated with propranolol (6 mg/kg). Ratio of these two concentrations, obtained from dry tissue weight, served as an index to intrarenal sodium gradient.

Propranolol inhibits the recovery of intrarenal sodium concentration gradient (Figure 1). Cortical sodium concentration remained constant. Medullary hypertonicity remained lower much longer in propranolol-treated animals. Three hours after frusemide injection, the papillary Na-cortical Na ratio was $3.6 \pm 0.2$ ($n=10$) instead of $4.4 \pm 0.2$ ($P < 0.05$) without propranolol.

Our grateful thanks to ICI for the (±)-propranolol hydrochloride.

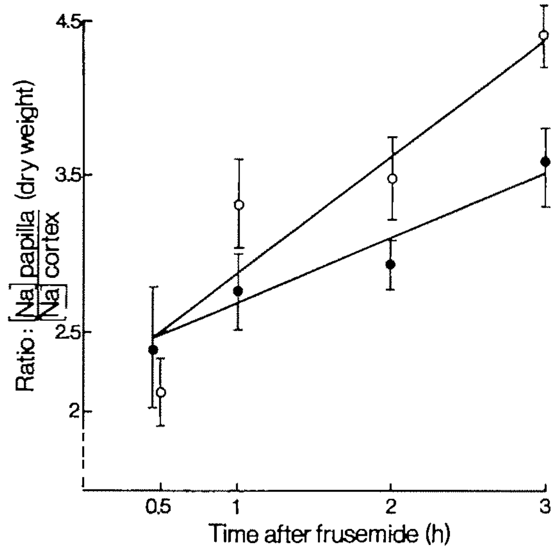


**Figure 1** Regression curves for the papillary Na/cortical Na ratio expressed as a function of time between frusemide injection and kidneys removal. The equation for the curve is $Y=0.797\ X+2.039$ ($r=0.6819$, $n=40$) after frusemide (○), and $Y=0.479\ X+2.184$ ($r=0.5233$, $n=40$) after frusemide combined with propranolol (●). The slopes of these 2 curves are significantly different ($P < 0.05$). Points represent the mean of 10 measurements, vertical bars, s.e. mean.

## References

ATHERTON, J.C., HAI, M.A. & THOMAS, S. (1968). The time course of changes in renal tissue composition during mannitol diuresis in the rat. *J. Physiol. (Lond.)*, **197**, 411–428.

IMBS, J.L., SCHMIDT, M., BELHADJ-MOSTEFA, H. & SCHWARTZ, J. (1975). Effet du dl-propranolol sur l'osmolarité urinaire après furosémide, chez le chien anesthésié. *C. R. Soc. Biol.*, **169**, 1365–1370.

---

# The role of potassium in the inhibition by cardiac glycosides of ($Na^+$-$K^+$)-ATPase prepared from human heart

A. DE POVER & T. GODFRAIND

*Université Catholique de Louvain, Laboratoire de Pharmacodynamie Générale et de Pharmacologie, Avenue E. Mounier, 73—UCL 7350, 1200 Bruxelles*

We have prepared an enriched fraction of human heart ($Na^+$-$K^+$)-ATPase from homogenates treated by NaI (De Pover & Godfraind, 1976). The activity of this preparation was equal to 10–15 μmol Pi mg protein$^{-1}$ h$^{-1}$ at 37°C in a medium containing (mM): NaCl 100, KCl 3, $MgCl_2$ 3, Tris-ATP 2.5, EGTA 1, Tris-maleate 20 (pH 7.4) (final volume 1 ml). The ATPase reaction was started by the addition of enzyme preparation (10 μg) and stopped by 0.1 ml of 50% trichloroacetic acid. The preparation contained 5% of residual Mg-ATPase.

The inhibition of ($Na^+$-$K^+$)-ATPase by digoxin, digitoxin, gitoxin and ouabain has been studied. The presence of the sugar chain and of the unsaturated lactone ring was required for full activity as reported

**Table 1** Concentrations of cardiac glycosides producing 50% inhibition of human heart ($Na^+$-$K^+$)-ATPase

| *Cardiac glycosides* | *$IC_{50}$ without preincubation in the absence of $K^+$ (μM)* | *$IC_{50}$ with preincubation in the absence of $K^+$ (μM)* |
|---|---|---|
| Digoxigenin | 0.36 (0.08) | 0.32 (0.03) |
| Digoxigenin digitoxoside | 0.066 (0.006) | 0.013 (0.001) |
| Digoxigenin *bis*-digitoxoside | 0.10 (0.01) | 0.020 (0.003) |
| Digoxin | 0.19 (0.02) | 0.036 (0.003) |
| Desacetyl lanatoside C | 0.30 (0.05) | 0.042 (0.004) |
| Dihydrodigoxin | 2.1 (0.3) | 2.6 (0.6) |

The activity of ($Na^+$-$K^+$)-ATPase was estimated by measuring the release of Pi after 1 h incubation at 37 °C. The incubation medium contained (mM) Na 100, KCl 10, $MgCl_2$ 3, Tris-ATP 2.5, EGTA 1, Tris-maleate 20 (pH 7.4) and various concentrations of cardiac glycosides.

In the experiments quoted without preincubation, the ATPase reaction was started by the addition of the enzyme to the incubation medium. In those quoted with preincubation, the enzyme preparation was preincubated for 15 min in the incubation medium where KCl was absent; the ATPase reaction was started by the addition of KCl to reach 10 mM.

$IC_{50}$ were estimated from dose effect curves. The standard error, given between brackets, was the estimated residual standard error of the straight line drawn by regression analysis with three concentrations ($n = 6$) which evoked an inhibition of between 20 and 80 per cent.

by Portius & Repke (1964) for guinea-pig heart ($Na^+$-$K^+$)-ATPase. As shown for the digoxin series (Table 1), the number of sugars was also important, the monoside being the most active compound as demonstrated with enzymes from animal species (Erdmann & Schöner, 1974).

In the presence of KCl concentrations varying from 0.5 to 20 mM, the dose-effect curve of digoxin was displaced to the right. Hunter-Downs plot analysis indicated competitive inhibition at potassium concentrations up to 5 mM and non-competitive inhibition above that concentration.

The concentrations of cardiac glycosides producing 50% inhibition of ($Na^+$-$K^+$)-ATPase ($IC_{50}$) were estimated with or without a preincubation in the absence of KCl (Table 1). As far as concerned digoxin and other heterosides, $IC_{50}$ measured with preincubation were 5 to 7 times lower than without preincubation ($P < 0.001$). This was not the case for digoxigenin nor for dihydrodigoxin; this indicates that their binding and the binding of the heteroside to the enzyme were dissimilar.

It has been shown that, within the range of free concentrations found in the blood of patients treated for heart failure ($10^{-9}$ M to $5.10^{-9}$ M), digitoxin stimulated the sodium pump in human heart slices and inhibited isolated ($Na^+$-$K^+$)-ATPase only by about 5 to 10% (Godfraind, 1972). Within this therapeutic range of concentrations, the cardiac glycosides here examined did not evoke an inhibition of ($Na^+$-$K^+$)-ATPase much different from that achieved by digitoxin.

This work has been supported by the F.R.S.M. (Contract No. 20121). We thank Dr C. Chalant and Dr J. Jaumin for providing us with human heart samples.

### References

DE POVER, A. & GODFRAIND, T. (1976). Sensitivity to cardiac glycosides of (Na + K)-ATPase prepared from human heart, guinea-pig heart and guinea-pig brain. *Arch. Int. Pharmacodyn. Ther.*, **221**, 339–341.

ERDMANN, E. & SCHÖNER, W. (1974). Ouabain-receptor interactions in (Na + K)-ATPase preparations. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **283**, 335–356.

GODFRAIND, T. (1972). Pharmacologie des récepteurs digitaliques. *Bull. Acad. Roy. Med. Belg.*, **12**, 403–443.

PORTIUS, H.J. & REPKE, K. (1964). Versuch einer Analyse der beziehungen zwischen chemischer Struktur und Digitalis-ähnlicher Wirksamkeit auf der Rezeptorebene. *Arzneim.-Forsch. (Drug Res.)*, **14**, 1073–1077.

# Batrachotoxin: activity-dependent prolongation of the cardiac action potential and positive inotropic effect

P. HONERJÄGER & M. REITER (introduced by U. TRENDELENBURG)

*Institut für Pharmakologie und Toxikologie der Technischen Universität München, Biedersteiner Str. 29, 8000 München 40*

Two steroid alkaloids, veratridine and batrachotoxin (BTX), are known to increase $P_{Na}$ of excitable membranes. In the case of veratridine, this effect is thought to underlie its positive inotropic action; increased passive Na influx will increase $[Na]_i$, Na/Ca exchange and therefore net Ca influx (Honerjäger & Reiter, 1975). We have examined (i) whether BTX increases $P_{Na}$ of guinea-pig myocardial cells and (ii) whether BTX has a positive inotropic effect analogous to that of veratridine. Our results confirm and extend some of the findings published recently by Shotzberger, Albuquerque & Daly (1976), but are at variance with their observations on the effect of BTX on the resting membrane potential of ventricular myocardium.

Six to ten minutes after addition of BTX (0.7 nM) to papillary muscles contracting at a rate of 1 Hz, the duration of the action potential starts to increase. Unlike veratridine, BTX prolongs the action potential at a progressively increasing rate. Quantitatively, this rate is proportional to the effect itself, suggesting that positive feedback is involved in the action of BTX. Twenty to forty minutes after addition of the alkaloid the prolonged repolarization phase gives rise to coupled extrasystoles. The effect on action potential duration is abolished by tetrodotoxin (10 µM) and largely reversed by washing with alkaloid-free solution (for 5 h) or by allowing the preparation to rest in the presence of BTX (for 2 h). The transient *hyper*polarization of the resting potential (Glitsch, 1973) which is observed after cessation of stimulation (1 Hz for $\geqslant 5$ min) is increased from $1.55 \pm 0.16$ mV to $3.72 \pm 0.36$ mV by BTX ($P < 0.001$).

The first action potential elicited after exposing a resting muscle for 30 min to BTX is not prolonged, no matter whether the muscle has been kept under control conditions ($[K]_o = 5.9$ mM), has been accumulating Na ($[K]_o = 0$) or has been depolarized ($[K]_o = 145.9$ mM). The 'slow response' ($[K]_o = 24.0$ mM; 1 Hz) is not prolonged during 30 min of exposure to BTX, neither is the first action potential elicited after return to 5.9 mM $[K]_o$. In each case, however, the action potential is prolonged during subsequent stimulation at 1 Hz and 5.9 mM $[K]_o$.

Concomitantly with its effect on action potential duration (but in the absence of a marked change of resting potential) BTX produces a positive inotropic effect by increasing rate of force development and prolongs relaxation time. These effects persist in preparations from reserpine-pretreated animals, but are prevented in muscles pretreated with tetrodotoxin. If stimulation is interrupted, the positive inotropic effect decays much faster than the effect on action potential duration. The positive inotropic effect of BTX is significantly enhanced under conditions known to inhibit Na extrusion (dihydro-ouabain, 20 µM; $[K]_o$ reduced to 2.0 mM).

In conclusion, our results suggest: (i) BTX prolongs the cardiac action potential by keeping Na channels open. This increases passive Na influx, $[Na]_i$ and Na extrusion as suggested by increased electrogenic Na pumping. The BTX-membrane interaction appears to require conditions which are known to be associated with the repetitive activation of fast Na channels. (ii) The effect of BTX on sarcolemmal $P_{Na}$ is responsible for its positive inotropic effect, but the relation is indirect. The probable link is increased $[Na]_i$ leading to increased Na/Ca exchange.

The authors are indebted to Drs Bernhard Witkop and John W. Daly for the gift of batrachotoxin.

## References

GLITSCH, H.G. (1973). An effect of the electrogenic sodium pump on the membrane potential in beating guinea-pig atria. *Pflügers Arch.*, **344**, 169–180.

HONERJÄGER, P. & REITER, M. (1975). The relation between the effects of veratridine on action potential and contraction in mammalian ventricular myocardium. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **289**, 1–28.

SHOTZBERGER, G.S., ALBUQUERQUE, E.X. & DALY, J.W. (1976). The effects of batrachotoxin on cat papillary muscle. *J. Pharmac. exp. Ther.*, **196**, 433–444.

# Inhibition of the stimulant effect of 5-hydroxytryptamine on cardiac sympathetic nerves by 5-hydroxytryptamine and related compounds

J.R. FOZARD & A.T.M. MOBAROK ALI

*Department of Pharmacology, Materia Medica and Therapeutics, The University, Manchester M13 9PT*

5-Hydroxytryptamine (5-HT) stimulates noradrenaline release from isolated rabbit hearts by activating tryptamine receptors on the terminal sympathetic nerves (Fozard & Mobarok Ali, 1976; Fozard & Mwaluko, 1976). A characteristic feature of the response is the tendency to rapid and reversible tachyphylaxis (Fozard & Mwaluko, 1976), which, in the absence of selective antagonists (Fozard & Mwaluko, 1975), is an important criterion for mediation through tryptamine receptors (Fozard & Mobarok Ali, 1976). This report explores this phenomenon further and details the potencies and selectivity of 5-HT and four structurally related compounds as antagonists of the stimulant effects of 5-HT on the cardiac sympathetic nerves.

Table 1

| *Compound* | *$pA_2$*† | *Molar concentration ratio (Inhibition)*<br>*Antilog (mean $pA_2$ 5-HT-mean $pA_2$ drug)* | *Molar dose ratio*‡ *(excitation)*<br>*$ED_{50}$ drug/$ED_{50}$ 5-HT* |
|---|---|---|---|
| 5-HT | 6.75 ± 0.11 (6) | 1 | 1 |
| *N*-methyl-5-HT | 6.48 ± 0.10 (3) | 1.7 | 2.2 (1.7–2.8) (4) |
| *N,N*-dimethyl-5-HT | 7.06 ± 0.10 (5) | 0.5 | 2.3 (1.8–3.0) (5) |
| 5,6-dihydroxytryptamine | 6.66 ± 0.15 (4) | 1.3 | 4.7 (3.2–6.9) (3) |
| tryptamine | 4.98 ± 0.09 (4) | 53 | 302* (135–676) (4) |

* Molar dose ratio $ED_{25}$ drug/$ED_{25}$ 5-HT.

† Mean values with standard errors.

‡ Mean values with 95% confidence limits (from Fozard & Mobarok Ali, 1976).

Hearts were removed from rabbits given heparin (500 u/kg) 5 min before killing and perfused at constant pressure by the Langendorff technique with Tyrode solution containing atropine (0.5 µg/ml) at 37°C. Right atrial and ventricular tensions and cardiac rate were recorded as previously described (Fozard & Muscholl, 1971). Drugs were given by bolus injection or incorporated into the perfusion fluid.

Cardiac stimulant responses to bolus injections of 5-HT (0.5–128 µg) were inhibited, concentration dependently, by perfusion of hearts with 5-HT (78 and 312 ng/ml), concentrations which did not themselves cause cardiac stimulation. The inhibition was selective in that responses to noradrenaline (0.04–40 µg) and dimethylphenylpiperazinium (5–320 µg) were unaffected.

Perfusion of hearts with tryptamine, 5,6-dihydroxytryptamine, *N*-methyl-5-HT, and *N,N*-dimethyl-5-HT which have been shown to activate neuronal tryptamine receptors (Fozard & Mobarok Ali, 1976) also inhibited responses to 5-HT. In each case, inhibition was selective in that responses to the $ED_{50}$ of noradrenaline and dimethylphenylpiperazinium were unaffected. Similarly, inhibition was produced without evidence for preliminary receptor stimulation. Using 5-HT as the agonist, the $pA_2$ values for the antagonist activities of 5-HT and its analogues were estimated by the method of Arunlakshana & Schild (1959) and are presented in Table 1. Also in Table 1 are the activities relative to 5-HT as excitants and inhibitors of the cardiac sympathetic neuronal tryptamine receptors.

The data suggest that 5-HT and several of its analogues with the property of stimulating neuronal tryptamine receptors can desensitize these receptors at concentrations lower than those required for excitation. If these experiments have physiological relevance, then it seems likely that neuronal inhibition would be the normally expected response since excitation occurs only at high concentrations or after bolus injection of the drugs.

**References**

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Brit. J. Pharmacol.*, **14**, 48–58.

FOZARD, J.R. & MOBAROK ALI, A.T.M. (1976). Evidence for tryptamine receptors on cardiac sympathetic nerves. *Br. J. Pharmac.* (in press).

FOZARD, J.R. & MUSCHOLL, E. (1971). A useful muscarinic parameter and the differential recording of atrial and ventricular tension in the perfused rabbit heart. *Naunyn-Schmiedebergs Arch. Pharmak.*, **270**, 319–325.

FOZARD, J.R. & MWALUKO, G.M.P. (1975). The stimulant response of 5-hydroxytryptamine on cardiac sympathetic nerves: resistance to blockade by classical tryptamine receptor antagonists. *Proc. 6th Int. Congr. of Pharmac., July 20–25, 1975, Helsinki, Finland*, Abstract 697.

FOZARD, J.R. & MWALUKO, G.M.P. (1976). Mechanism of the indirect sympathomimetic effect of 5-hydroxytryptamine on the isolated heart of the rabbit. *Br. J. Pharmac.*, **57**, 115–125.

---

## Effects of carbonic anhydrase inhibitors upon cerebral cortex oxygen availability and resistance to hypoxia

R.G. BOULU, R.G. LARSEN,
M. PLOTKINE & P. ROSSIGNOL

*Laboratoire de Pharmacodynamie, Faculté des Sciences Pharmaceutiques et Biologiques, Université René-Descartes, Paris*

Acetazolamide has been used in the treatment of respiratory insufficiency and appears to aid accommodation to altitude in man. This drug is able to increase cerebral blood flow without influencing the cerebral metabolic rate of oxygen. This effect seems to be due to raised $pCO_2$ of brain tissue (Gotoh, Meyer & Tomita, 1966).

The influence of carbonic anhydrase inhibitors (acetazolamide, methazolamide, dichlorphenamide) upon cerebral tissue $pO_2$ has been studied in unanaesthetized rabbits with chronically implanted oxygen electrodes. Intravenous administration of these drugs (5–25 mg/kg) rapidly induced a significant and long-lasting rise of cerebral $pO_2$. Meanwhile, the $pO_2$ response to $CO_2$ inhalation was not reduced.

Resistance to atmospheric decompression in mice was increased by carbonic anhydrase inhibitors, but these drugs displayed no protection against asphyxial hypoxia in rats.

These results are in accordance with the reported clinical data.

### Reference

GOTOH, F., MEYER, J.S. & TOMITA, M. (1966). Carbonic anhydrase inhibition and cerebral venous blood gases and ions in man. *Arch. Intern. Med.*, **117**, 39–46.

---

## Long-lasting effects of the opening of the blood brain barrier on the modifications induced by intracarotid injection of noradrenaline

F.R. BOISMARE, L. HACPILLE,
J.P. LEFRANCOIS & M. LEPONCIN

*Department of Pharmacology, Hotel-Dieu, rue de Lecat, 76038—Rouen, France*

There are three problems concerning the haemodynamic effects of noradrenaline: (1) Does noradrenaline cross the blood brain barrier, when injected into the carotid artery? (2) What are the effects of such an injection on cerebral blood flow, whether the blood brain barrier is opened or not? (3) Which haemodynamic effects might be considered to be of central origin?

The following observations were made on dogs: (1) Vertebral blood flow was decreased after the intracarotid injection of noradrenaline. Thus, the vertebral artery is responsive to noradrenaline. After the blood–brain barrier had been opened (urea 36%, 10 ml), the decrease of the vertebral blood flow was more marked and so was related to the effects of noradrenaline itself on the vertebral artery and/or its underlying system. (2) Cardiac output increased immediately after the injection of noradrenaline subsequent to the first injection of urea. Thus, there must be central cardiostimulating structures responsive to noradrenaline. (3) Mean arterial blood pressure, total peripheral resistance and heart rate decreased, and femoral blood flow increased only after the injection of noradrenaline given after a second injection of urea.

Hence, the bradycardia and the increase of cardiac output, which are both centrally mediated, are therefore related to two different mechanisms, since noradrenaline increased cardiac output immediately after the first injection of urea, but induced bradycardia only after the second injection of urea. Thus, the effects of noradrenaline can be modulated by modifications of the permeability of the blood brain barrier.

# Neuronal responses to adrenoceptor agonists in the cerebral cortex: evidence for excitatory α-adrenoceptors and inhibitory β-adrenoceptors

P. BEVAN, C.M. BRADSHAW &
E. SZABADI

*Department of Psychiatry, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT*

Single cortical neurones are sensitive to noradrenaline (NA) applied by microelectrophoresis; both excitatory and depressant responses have been described (Krnjević & Phillis, 1963; Johnson, Roberts, Sobieszek & Straughan, 1969; Bevan, Bradshaw, Roberts & Szabadi, 1974). In the present study we have compared the effects of NA with those of other α- and β-adrenoceptor agonists on cortical neurones.

Spontaneously active single neurones were studied in the somatosensory cortex of the halothane-anaesthetized rat. All the drugs were applied by microelectrophoresis. Our techniques have been described elsewhere (Bevan *et al.*, 1974).

Out of 194 cells giving consistent responses to NA, 128 (66%) were excited and 66 (34%) were depressed by the drugs. Out of 93 cells responding to isoprenaline (IPNA), 26 (28%) were excited, and 67 (72%) were depressed. IPNA was applied with increasing ejecting currents (0–100 nA) to 22 cells which were depressed when IPNA was applied with lower ejecting currents; on 14 cells the depression was reversed into an excitation as the intensity of the ejecting current was increased. Phenylephrine was studied on 23 cells; 22 of these cells gave consistent excitatory responses, one cell did not respond. Salbutamol was studied on 11 cells excited by phenylephrine; all these cells were depressed by salbutamol.

Excitatory responses to NA were reversibly antagonized by phentolamine (10 cells), phenoxybenzamine (5 cells), and propranolol (4 cells). Excitatory responses to IPNA could be antagonized by phenoxybenzamine (5 cells); on one of these cells a depressant response was revealed after the abolition of the excitation. Excitatory responses to IPNA were reversibly antagonized by sotalol on two cells. Excitatory responses to phenylephrine were reversibly antagonized by phenoxybenzamine (7 cells); on two of these cells the depressant response to IPNA, and on one cell the depressant response to salbutamol, was not affected by phenoxybenzamine. Acetylcholine (ACh) was used as a control agonist in each of the antagonism studies: responses to ACh were not affected.

Depressant responses to IPNA could be antagonized by sotalol (10 cells). On two of these cells an excitation was revealed after the abolition of the depressant response. Responses to ACh were not affected.

These results indicate that the same cortical neurone may respond both with excitation and depression to adrenoceptor agonists; the excitatory responses may be mediated by α- and the depressant responses by β-adrenoceptors. The ability of the β-adrenoceptor antagonists (propranolol, sotalol) to antagonize excitatory responses (see also Johnson *et al.*, 1969) could reflect the α-adrenoceptor blocking properties of these drugs at somewhat higher concentrations than are required for β-adrenoceptor blockade (Gulati, Gokhale, Parikh, Udwadia & Krishnamurty, 1969). Indeed, lower doses of sotalol often potentiate nueronal excitatory responses to NA, whereas higher doses have an antagonistic effect (Bevan *et al.*, 1974).

This work was supported by the North Western Regional Health Authority, and the Mental Health Trust and Research Fund.

**References**

BEVAN, P., BRADSHAW, C.M., ROBERTS, M.H.T. & SZABADI, E. (1974). The effect of microelectrophoretically applied mescaline on cortical neurones. *Neuropharmacology*, **13**, 1033–1045.

GULATI, O.D., GOKHALE, S.D., PARIKH, H.M., UDWADIA, B.P. & KRISHAMURTY, V.S.R. (1969). Evidence for a sympathetic *alpha* receptor blocking action of *beta* receptor blocking agents. *J. Pharm. exp. Ther.*, **166**, 35–43.

JOHNSON, E.S., ROBERTS, M.H.T., SOBIESZEK, A. & STRAUGHAN, D.W. (1969). Noradrenaline sensitive cells in cat cerebral cortex. *Int. J. Neuropharmac.*, **8**, 549–566.

KRNJEVIĆ, K. & PHILLIS, J.W. (1963). Actions of certain amines on cerebral cortical neurones. *Brit. J. Pharmacol.*, **20**, 471–490.

## Delta-9-tetrahydrocannabinol (THC) and macromolecular synthesis: mechanisms of action

B. DESOIZE, J.C. JARDILLIER,
C. LEGER & G.G. NAHAS

*Laboratoire de Pharmacologie et de Toxicologie Cellulaires INSERM U-26, 200 rue du Faubourg St Denis, 75010 Paris*

We have reported (Nahas, Desoize, Armand & Morishima, 1975) that in $10^{-5}$ to $10^{-4}$ M concentration, THC inhibits the incorporation of [$^3$H]-leucine, [$^3$H]-uridine and [$^3$H]-thymidine in cultured human lymphocytes stimulated with phytohemagglutinin (PHA). The present experiments were designed to clarify the mechanism of action of this depressant effect of THC on blastogenesis.

First, the time course of this effect of THC was studied. We observed that within 15 min after its addition, THC inhibited the incorporation of three precursors of protein, RNA and DNA: leucine, uridine and thymidine. This data indicates that THC does not exert its inhibitory effect specifically on the synthesis of a single macromolecule; such a type of specific inhibition is characterized by a lag in the synthesis of the macromolecules that are not primarily affected by the inhibition. In the present experiments, cell synthesis of all three macromolecules was inhibited at the same time.

In a second series of experiments we measured the effect of THC on uptake of leucine, uridine and thymidine into the intracellular pool. This uptake was significantly inhibited by the drug. This inhibition of precursor uptake in the cellular pool can explain the impairment of macromolecular synthesis.

Pool formation of precursors may be inhibited by three mechanisms which were next studied. Inhibition of transport enzymes, increased cellular 'leaking', or inhibition of ATP synthesis. Cellular 'leaking' was tested by incubating for 2 h lymphocytes with [$^{14}$C]-aminoisobutyric acid (a substance which is transported into the cell, but not incorporated into macromolecules); the cells were then washed, incubated for 30 min with THC and centrifuged; radioactivity was measured in the supernatant; it was not different from that obtained in control preparations. Effect of THC on cellular energy production was tested by: (a) measuring effect of THC on lymphocyte anaerobic glycolysis (glucose consumption and lactate production): it was not affected by the drug; (b) measuring cellular ATP concentration: there was no significant decrease compared with control cultures.

It would therefore appear that THC exerts its inhibitory effect on macromolecular synthesis to a greater extent by interfering with the transport enzymes of the plasma membrane. THC is known to bind on the plasma membrane (Chari-Bitron, 1971). At the level of the plasma membrane THC might act directly by altering its physico-chemical characteristics (Seeman, 1972). THC would therefore exert its inhibitory effect on cell anabolism during every stage of the cell cycle.

### References

CHARI-BITRON, A. (1971). Stabilization of rat erythrocyte membrane by delta-1-tetrahydrocannabinol. *Life Sci.*, **10**, 1273–1279.

NAHAS, G.G., DESOIZE, B., ARMAND, J.P. & MORISHIMA, A. (1975). Inhibition par les cannabinoïdes naturels de la transformation lymphocytaire et de la synthèse des acides nucléiques. *J. Pharmacol.*, **6**, 377.

SEEMAN, P. (1972). The membrane actions of anesthesics and tranquilizers. *Pharmac. Rev.*, **24**, 583–655.

---

## Is the gastric antisecretory property of clonidine in rats of central origin?

X.B. PASCAUD & A.R. ROGER

*Department of Pharmacology—I.N.R.S., 14 rue du Val d'Or—92150 Suresnes, France*

It seems now established that even though clonidine is ineffective (Boissier, Giudicelli, Larno & Fichelle, 1970) or even stimulates the gastric secretion of anaesthetized rats as it does in pentagastrin-stimulated Heidenhain pouch dogs (Curwain & Endersby, 1974), it possesses powerful gastric antisecretory properties in conscious rats (Walz & Van Zwieten, 1970) and in man (Kaess & Von Mikulicz-Radecki, 1971). This report describes the effects of clonidine in the 4 h-pylorus ligated rat and in basal or stimulated gastric secretion in chronic fistula rats in order to verify the hypothesis that this action is probably of central origin.

Dose response curves were established in male Sprague–Dawley rats (250–300 g) either by the 4 h-pylorus ligated Shay rat preparation or in 2 × 2 h basal secretion tests in chronic gastric fistula rats (Pascaud, Errard & Blouin, 1974), some of the latter bearing stereotaxically implanted micro-cannula in the lateral ventricle of the brain. On rats with gastric fistulae, the effect of clonidine (30 µg/kg s.c.) was determined on acid secretion stimulated by continuous i.v. infusions (2 ml/h) in a tail vein of histamine (1 mg kg$^{-1}$ h$^{-1}$), pentagastrin (6 µg kg$^{-1}$ h$^{-1}$), 2 deoxy-*d*-glucose (2 DG, 25 mg kg$^{-1}$ h$^{-1}$) and methacholine (0.6 mg kg$^{-1}$ h$^{-1}$). Total acidity was automatically titrated at $pH = 8.5$ (ATS$_1$, Radiometer).

Gastric acid output decreased markedly after clonidine in both pylorus ligated and chronic fistula rats under basal conditions. The corresponding ED$_{50}$s were 27 (15–48) µg/kg, i.p., and 13.6 (5.4–34) µg/kg, s.c. respectively. In contrast the ED$_{50}$ was 5.8 (1.6–21) µg/kg when clonidine was administered in the lateral ventricle of the brain. Furthermore clonidine (30 µg/kg s.c.) produced a very significant ($P < 0.01$) and long lasting (>3 h) inhibition when maximal acid responses were obtained with histamine, pentagastrin or 2 DG infusion. On the contrary no inhibition was seen on the secretion stimulated by methacholine.

Thus clonidine used in the same dose range as that described in dogs for its cardiovascular activities possesses powerful gastric antisecretory properties in basal and stimulated acid secretion of conscious rats. Considering first the marked inhibition obtained on the 2 DG stimulation and the lack of activity on the methacholine, and second the very low ED$_{50}$ registered when clonidine was administered in the lateral ventricle of the brain, it seems very likely that its gastric antisecretory property is of central origin in rats. Work is now in progress to determine more precisely the nature of the central neurone receptors involved in this activity.

**References**

BOISSIER, J.R., GIUDICELLI, J.F., LARNO, S. & FICHELLE, J. (1970). Action de la Clonidine (ST 155) sur la sécrétion gastrique et l'ulcère expérimental. *J. Pharmacol.*, **1**, 109–122.

CURWAIN, B.P. & ENDERSBY, K. (1974). Enhancement of pentagastrin induced gastric acid secretion by clonidine in the conscious dog. *Br. J. Pharmac.*, **50**, 431–434.

KAESS, H. & VON MIKULICZ-RADECKI, J. (1971). Der Einfluss von 2-(2,6-Dichlorphenylamino)-2-imidazolin-hydrochlorid (Catapresan ) auf die funktion von Magen und Pancreas. *Europ. J. clin. Pharmacol.*, **3**, 97–101.

PASCAUD, X.B., ERRARD, D.J. & BLOUIN, M.M. (1974). The gastric antisecretory activity of Pyridyl-2-Thioacetamide (CMN 131). *Am. J. Dig. Diseases*, **19**, 503–515.

WALZ, A. & VAN ZWIETEN, P.A. (1970). The influence of 2-(2,6-Dichlorophenylamino)-2-Imidazoline hydrochloride (Clonidine) and some related compounds on gastric secretion in the anaesthetized rat. *Eur. J. Pharmac.*, **10**, 369–377.

---

# Effects of *p*-chloro-β-phenylethylamine on the uptake and release of putative amine neurotransmitters in rat brain

G.B. BAKER, A. BERTOLLINI,
R. del CARMINE, I.L. MARTIN &
M. RAITERI

*MRC Neuropharmacology Unit, The Medical School, Birmingham, UK and Istituto di Farmacologia, Universita Cattolica, Roma, Italy*

The monoamine *p*-chloro-β-phenylethylamine (PCPE), a metabolite of *p*-chlorophenylalanine (PCPA) has been reported by Koe & Weissman (1966) to cause a decrease in brain noradrenaline (NA) and 5-hydroxytryptamine (5-HT) levels following its injection intraperitoneally into rats. Our findings in littermate male Wistar rats, using the assay procedure of Martin & Ansell (1973), are in agreement with this. NA levels were 74.8 ± 3.5% and 5-HT levels 74.7 ± 4.8% of controls (mean ± s.e. mean, $n = 6$) 15 min after PCPE, 40 mg/kg i.p. In the same animals we found no significant change in dopamine (DA) levels from controls.

We have now investigated the effects of PCPE on uptake and release of $^3$H-labelled DA, NA and 5-HT *in vitro* using methods previously described (Raiteri, Angelini & Levi, 1974; Raiteri, Levi & Federico, 1974). Crude synaptosomal P$_2$ fractions (Gray & Whittaker, 1962) prepared from rat hypothalamus (NA studies) or striatum (DA and 5-HT studies) were used, and all incubation and superfusion media contained nialamide ($1.25 \times 10^{-5}$ M).

PCPE was a stronger inhibitor of 5-HT and NA uptake than of DA uptake (IC$_{50} = 2.2 \times 10^{-6}$ M, $5.0 \times 10^{-6}$ M and $1.5 \times 10^{-5}$ M respectively) and, as shown in Figure 1, showed marked differences in its effect on the release of the three transmitters, being a particularly strong stimulator of 5-HT release. Simultaneous experiments with β-phenylethylamine demonstrate that addition of the *p*-chloro substituent on β-phenylethylamine has little effect on NA release, but has a dramatic influence on 5-HT and DA release, increasing the former and decreasing the latter.

This compound appears to be a useful analogue for studying structure-activity relationships in the effects of phenylethylamine derivatives on the transport of amine neurotransmitters *in vivo* and *in vitro*, although to maintain substantial levels *in vivo* for long periods

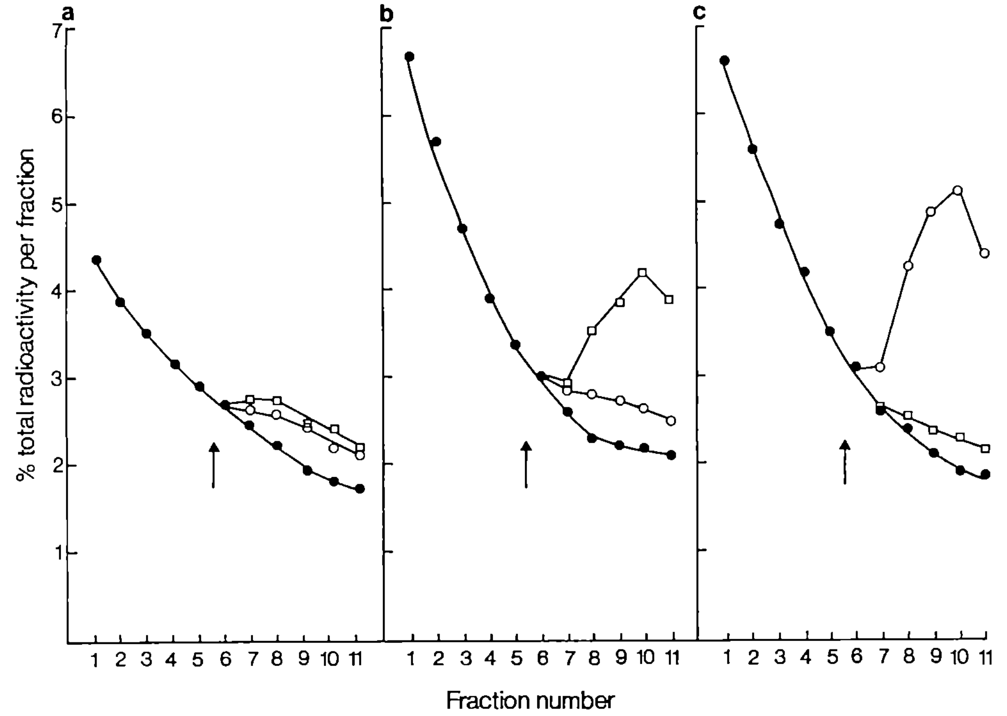


**Figure 1** Effect of *p*-chloro-*β*-phenylethylamine (PCPE) and *β*-phenylethylamine (PE) on biogenic amine release. $P_2$ preparations, prelabelled with tritiated noradrenaline (NA), dopamine (DA) or 5-hydroxytryptamine (5-HT) were collected on millipore filters in superfusion chambers and superfused at a rate of 0.5 ml/minute. Fractions of the superfusate were collected at 1 min intervals. The radioactivity of each fraction is expressed as a percentage of the total radioactivity recovered (fractions plus filter) at the end of the superfusion period. The arrow indicates the time of addition of PCPE or PE (final concentration, $10^{-6}$ M). Each curve is the mean of 3 experiments run in triplicate, and PCPE and PE were tested simultaneously, in parallel superfusion chambers. (a) Hypothalamus, $^3$H-noradrenaline release; (b) corpus striatum, $^3$H-dopamine release, (c) corpus striatum, $^3$H-5-hydroxytryptamine release, ●, control; ○, PCPE; □, PE.

following injection of PCPA or PCPE, it is necessary to do experiments in conjunction with a monoamine oxidase (MAO) inhibitor.

G.B.B. is a Fellow of the Canadian MRC.

**References**

GRAY, E.G. & WHITTAKER, V.P. (1962). The isolation of nerve endings from brain: an electron microscope study of cell fragments derived by homogenization and centrifugation. *J. Anat. (Lond.)*, **96**, 79–87.

KOE, B.K. & WEISSMAN, A. (1966). *p*-Chlorophenylalanine, a specific depletor of brain serotonin. *J. Pharmac. exp. Ther.*, **154**, 499–516.

MARTIN, I.L. & ANSELL, G.B. (1973). A sensitive gas chromatographic procedure for the estimation of noradrenaline, dopamine and 5-hydroxytryptamine in rat brain. *Biochem. Pharmac.*, **22**, 521–533.

RAITERI, M., ANGELINI, F. & LEVI, G. (1974). A simple apparatus for studying the release of neurotransmitters from synaptosomes. *Eur. J. Pharmac.*, **25**, 411–414.

RAITERI, M., LEVI, G. & FEDERICO, R. (1974). *d*-Amphetamine and the release of $^3$H-norepinephrine from synaptosomes. *Eur. J. Pharmac.*, **28**, 237–240.

# Modification by oestrogen of the effects of (D)-amphetamine sulphate on noradrenaline metabolism in discrete areas of rat brain

JOAN M. FLUDDER & SALLY R. TONGE
(introduced by B.E. LEONARD)

*School of Pharmacy, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, England*

The changes in brain monoamine concentrations which accompany hormonal changes in female animals may be partially explained as direct effects of ovarian steroids on central monoamine metabolism (Greengrass & Tonge, 1974). Fludder & Tonge (1975) have shown changes in monoamine concentrations in eight areas of rat brain during the oestrous cycle which were expected to affect both behaviour and the effects of psychotropic drugs. The effects of endogenous oestrogen, of ethinyloestradiol, and of (+)-amphetamine sulphate in the eight areas previously described have now been examined in female rats at the four stages of the oestrous cycle and in litter-mates ovariectomized six weeks prior to use. To some extent, the effects seen were dependent upon

**Table 1** Effects of oestrogens and amphetamine on catecholamine concentrations in rat brain

| | *Dopamine (nmol/g ± s.e. mean)* | *cf.* | *Noradrenaline (nmol/g ± s.e. mean)* | *cf.* | *Normetanephrine (nmol/g ± s.e. mean)* | *cf.* |
|---|---|---|---|---|---|---|
| Dioestrus (Di., minimal oestrogen secretion) | | | | | | |
| amygdala | 0.60 ± 0.10 | | 2.64 ± 0.02 | | 0.64 ± 0.09 | |
| midbrain | 2.52 ± 0.10 | | 2.86 ± 0.02 | | 3.52 ± 0.09 | |
| Di. + Amphetamine (A, 10 mg/kg, i.p., cf. Di.) | | | | | | |
| amygdala | 1.12 ± 0.09 | (+) | 1.48 ± 0.01 | (–) | 2.04 ± 0.09 | (+) |
| midbrain | 3.10 ± 0.14 | (+) | 1.36 ± 0.02 | (–) | 5.28 ± 0.02 | (+) |
| Di. + ethinyloestradiol (EO, 100 μg/kg, s.c., cf. Di.) | | | | | | |
| amygdala | 0.64 ± 0.05 | (0) | 2.67 ± 0.07 | (0) | 0.65 ± 0.08 | (0) |
| midbrain | 3.23 ± 0.03 | (+) | 4.58 ± 0.15 | (+) | 3.80 ± 0.11 | (0) |
| Oestrus (Oe., maximal oestrogen secretion, cf. Di.) | | | | | | |
| amygdala | 2.80 ± 0.01 | (+) | 1.47 ± 0.05 | (–) | 2.23 ± 0.16 | (+) |
| midbrain | 1.75 ± 0.07 | (–) | 2.33 ± 0.10 | (–) | 4.35 ± 0.20 | (+) |
| Oe. + A (cf. Oe.) | | | | | | |
| amygdala | 0.38 ± 0.01 | (–) | 1.57 ± 0.04 | (0) | 2.06 ± 0.09 | (0) |
| midbrain | 1.53 ± 0.03 | (0) | 2.05 ± 0.01 | (0) | 3.25 ± 0.23 | (–) |
| Ovariectomized (Ov., 6 weeks prior to use, cf. Di. litter-mates) | | | | | | |
| amygdala | 1.61 ± 0.16 | (+) | 3.30 ± 0.26 | (+) | 7.31 ± 0.50 | (+) |
| midbrain | 2.94 ± 0.36 | (0) | 0.53 ± 0.08 | (+) | 2.77 ± 0.01 | (–) |
| Ov. + EO (cf. Ov.) | | | | | | |
| amygdala | 1.03 ± 0.24 | (–) | 1.78 ± 0.18 | (–) | 0.61 ± 0.06 | (–) |
| midbrain | 3.45 ± 0.48 | (0) | 0.86 ± 0.12 | (+) | 2.51 ± 0.01 | (0) |
| Ov. + A (cf. Ov.) | | | | | | |
| amygdala | 2.47 ± 0.47 | (+) | 3.38 ± 0.15 | (0) | 8.10 ± 0.33 | (0) |
| midbrain | 2.92 ± 0.05 | (0) | 0.45 ± 0.01 | (0) | 2.52 ± 0.10 | (0) |
| Ov. + EO + A (cf. Ov.) | | | | | | |
| amygdala | 2.39 ± 0.47 | (+) | 3.09 ± 0.19 | (+) | 0.81 ± 0.01 | (–) |
| midbrain | 4.36 ± 0.51 | (+) | 7.00 ± 0.35 | (+) | 2.01 ± 0.05 | (–) |
| Ov. + EO + A (cf. Ov + EO) | | | | | | |
| amygdala | | (+) | | (+) | | (+) |
| midbrain | | (+) | | (+) | | (–) |
| Ov. + EO + A (cf. Ov. + A) | | | | | | |
| amygdala | | (0) | | (0) | | (–) |
| midbrain | | (+) | | (+) | | (–) |

Only statistically significant changes ($P < 0.05$, Student's *t* test) are shown as increases (+) or decreases (–) in the '*cf.*' columns. There were at least 5 rats per group.

the relative preponderance of noradrenaline (NA) terminals or of NA axons/bodies in each brain area, suggesting that NA metabolism cannot be treated as a single, homogenous system in the CNS; the amygdala region is an example of an area in which terminals predominate, and the midbrain region of an area in which axons/bodies predominate. The effects of oestrogens and amphetamine are shown in Table 1.

NA depletion after synthesis blockade with $\alpha$-methyl-*p*-tyrosine (500 mg/kg, i.p.) was slower in ovariectomized than in dioestrous litter-mates, including a reduced NA turnover rate; depletion was accelerated by ethinyloestradiol. NA depletion after 4,$\alpha$-dimethyl-*m*-tyramine (2 x 12.5 mg/kg, i.p.), which reflects changes in NA neuronal uptake mechanisms, was antagonized in the amygdala, but not in the midbrain, regions by ethinyloestradiol.

It is suggested that both amphetamine and oestrogens release newly-synthesized NA, amphetamine by its well-established action of stimulating release from catecholamine terminals, whilst inhibiting impulse flow (Graham & Aghajanian, 1971) and oestrogens by stimulating total turnover of NA. Endogenous ovarian steroids appear to be necessary for the maintenance of 'normal' NA turnover rates; in their absence, or when oestrogen levels are high (at oestrus) so that maximal NA release is already occurring, amphetamine cannot show its expected effects.

**References**

FLUDDER, J.M. & TONGE, S.R. (1975). Variations in the concentrations of monoamines and their metabolites in eight regions of rat brain during the oestrous cycle: a basis for interactions between hormones and psychotropic drugs. *J. Pharm. Pharmac.*, **27** *Suppl.*, 39.

GRAHAM, A. & AGHAJANIAN, G.K. (1971). Effects of amphetamine on single cell activity in a catecholamine nucleus, the locus coeruleus. *Nature (Lond.)*, **234**, 100–103.

GREENGRASS, P.M. & TONGE, S.R. (1974). Suggestions on the pharmacological actions of ethinyloestradiol and progesterone on the control of monoamine metabolism in three regions from the brains of gonadectomized male and female mice, and the possible clinical significance. *Arch. int. pharmacodyn.*, **211**, 291–303.

---

## L-Dopa and (—)-deprenil in the treatment of Parkinson's disease: a long-term study

L. AMBROZI, W. BIRKMAYER,
P. RIEDERER & M.B.H. YOUDIM

*Ludwig Boltzmann Neurochemistry Institute, A-1130 Vienna-Lainz, Austria, and MRC Clinical Pharmacology Unit, Radcliffe Infirmary, Oxford OX2 6HE, UK*

L-Dopa plus a peripheral decarboxylase inhibitor has been used with some success in the treatment of Parkinson's Disease (Birkmayer, Linauer, Mentasti & Riederer, 1974). However, therapeutic success decreases as the illness progresses and various side effects, e.g. on-off effect, occur with this form of treatment.

In a recent study on Parkinsonian patients we demonstrated that deprenil (a selective inhibitor of monoamine oxidase (MAO) 'type B') can significantly potentiate the anti-akinetic effect of L-dopa in akinetic patients (Brikmayer, Riederer, Youdim & Linauer, 1975). Furthermore, the addition of deprenil results in a daily reduction of daily dose requirements of Madopar (L-dopa and the peripheral decarboxylase inhibitor of Benseracide (N-$^1$, DL-seryl-N-$^2$, (2, 3, 4-trihydroxybenzyl) hydrazine)). The aim of this study has been to examine the long-term effects of Madopar plus deprenil treatment in Parkinsonian patients.

Two hundred and twenty-three patients have been treated with Madopar plus deprenil since October 1974. The average oral dose of Madopar was 250 mg three times daily, and of deprenil 5–10 mg once daily. In a few patients 50 mg of L-dopa and 10 mg of deprenil were administered intravenously; in these patients intravenous therapy was more effective than oral therapy but side effects occurred more often and to a greater extent and this mode of treatment was therefore discontinued. As shown in Table 1 the addition of deprenil to Madopar therapy resulted in a statistically significant reduction in patients' functional disability (Birkmayer & Neumayer, 1972). Of the 223 patients, abnormal involuntary movements occurred in 16, psychosis in 14, orthostatic hypotension in 5 and nausea in eight. In patients with side-effects deprenil treatment was either terminated or reduced to 5 mg resulting in the disappearance of some of the side-effects. Madopar-deprenil therapy produced no response in 13.9% of patients.

The improvement in disability following deprenil therapy occurred within 20–120 min after a single dose and lasted for one to three days. Thus, deprenil may act not only by inhibiting MAO but also as a psycho-stimulant by releasing dopamine in a fashion similar to amphetamine (Knoll, Ecseri, Kelemen, Nievel & Knoll, 1965; Fuxe & Ungerstedt, 1970; Christmas, Coulson, Maxwell & Riddell, 1972).

This study has shown that the addition of deprenil

**Table 1** Improvement of the disability after Madopar and Madopar plus (−)-deprenil treatment in Parkinsonian patients.

| *Duration of disease (yrs)* | *No.* | *Age (yrs)* | Percentage disability<br>*Before therapy* | | *After Madopar* | | *After Madopar + (−)-deprenil* |
|---|---|---|---|---|---|---|---|
| 0–6 | 115 (55♂, 60♀) | 68.5 ± 0.8 | 54.3 ± 1.5 | $P<0.001$ | 36.5 ± 1.4 | $P<0.001$ | 25.3 ± 1.3 |
| 7–15 | 108 (60♂, 48♀) | 69.4 ± 0.7 | 60.1 ± 1.3 | $P<0.001$ | 37.2 ± 1.4 | $P<0.01$ | 28.4 ± 1.4 |

Results are stated as mean ± s.e. mean.

to Madopar therapy results in an improvement of disability independent of the duration of the illness (Table 1) and suggests that the inclusion of deprenil leads to a better utilization of synthesized dopamine from L-dopa.

### References

BIRKMAYER, W., LINAUER, W., MENTASTI, M. & RIEDERER, P. (1974). Zweijährige Erfahrungen einer Kombinationsbehandlung des Parkinson-syndroms mit L-dopa und dem Decarboxylasehemmer Benseracide (RO4–4602). *Wien Med. Wochenschr.*, **124**, 340–344.

BIRKMAYER, W. & NEUMAYER, E. (1972). Die moderne medikamentöse Behandlung des Parkinsonismus. *Z. Neurol.*, **202**, 257–280.

BIRKMAYER, W., RIEDERER, P., YOUDIM, M.B.H. & LINAUER, W. (1975). The potentiation of the anti-akinetic effect after L-dopa treatment by an inhibitor of MAO-B, Deprenil. *J. Neurol. Trans.*, **36**, 303–326.

CHRISTMAS, A.J., COULSON, C.J., MAXWELL, D.R. & RIDDELL, D. (1972). A comparison of the pharmacological and biochemical properties of substrate-selective monoamine oxidase inhibitors. *Br. J. Pharmac.*, **45**, 490–503.

FUXE, K. & UNGERSTEDT, U. (1970). Histochemical, biochemical and functional studies on central monoamine neurons after acute or chronic amphetamine administration. In *Amphetamines and Related Compounds*, eds. Costa, L. & Garattini, S., pp. 257–288. New York: Raven Press.

KNOLL, J., ECSERI, Z., KELEMEN, K., NIEVEL, J. & KNOLL, B. (1965). Phenylisopropylmethyl propinylamine (E-250), a new spectrum psychic energizer. *Arch. Int. Pharmacodyn.*, **155**, 154–164.

---

## Autoradiographic evidence for the effects of specific uptake-inhibitors on the selective accumulation of [$^3$H]-5-HT by supra-ependymal nerve terminals and for the localization of binding sites for [$^3$H]-D LSD

J.G. RICHARDS

*Pharmaceutical Research Department, F. Hoffmann-La Roche & Co., Ltd., Basle, Switzerland*

It can be shown, by light microscopic autoradiography, that intraventricular injection of tritium-labelled 5-hydroxytryptamine ([$^3$H]-5-HT; 70 µM) leads to its accumulation not only in the brain parenchyma in the rat but also on the ependymal surface of some regions, e.g. corpus callosum, nucleus caudatus and cranial floor of the fourth ventricle; electron microscopy of these regions reveals that the radiolabel is localized to supra-ependymal nerve terminals. The ependymal surface of regions which are known to lack these nerve terminals, e.g. hypothalamus ventralis anterior, eminentia mediana and roof (velum medullare) of the fourth ventricle, was free of label. Intraventricular administration of both [$^3$H]-dopamine (DA; 350 µM) and [$^3$H]-noradrenaline (85 µM) led to their accumulation in the parenchyma but not above the ependyma in all brain regions investigated, i.e. supra-ependymal nerve terminals were not labelled.

Chlorimipramine and reserpine, which block neuronal uptake and storage respectively in tryptaminergic nerves, prevented the accumulation of $[^3H]$-5-HT in supra-ependymal nerve terminals. However, desipramine, a known catecholamine-uptake blocker, had no effect on this accumulation.

The demonstration of a specific uptake mechanism for 5-HT in supra-ependymal nerve terminals confirms the tryptaminergic nature of these nerves (Richards, Lorez & Tranzer, 1973; Lorez & Richards, 1973; Richards & Tranzer, 1974; Lorez & Richards, 1975). The physiological significance of this uptake mechanism is likely to be the removal of the amine from the vicinity of the effector organ (periventricular target cell) in order to terminate the possible neurotransmitter action of 5-HT. The presence of 5-HT in CSF (Holman & Vogt, 1972), probably secreted in part by supra-ependymal nerve terminals, suggests that there may be specific receptors and a physiological role for this amine in periventricular brain regions. This may also be true of the human brain since supra-ependymal nerve terminals have recently been observed in the lateral and fourth ventricles of human postmortem tissue by electron microscopy; it might be expected that these nerves also store and accumulate 5-HT although this has to be demonstrated.

Since the hallucinogenic drug DLSD is known to have a high affinity for 5-HT (Bennett & Snyder, 1975) and DA (Creese, Burt & Snyder, 1976) receptors, the localization of binding sites for $[^3H]$-DLSD was studied by autoradiography in order to identify 5-HT receptors in periventricular brain regions (presuming the absence of DA receptors in these regions). The results indicate that, 1 h after intraventricular administration of 350 μM $[^3H]$-DLSD, a significant binding of LSD could be observed in the ependymal and subependymal zone of several periventricular brain regions. However, the label did not seem to be selectively bound to any particular structure. Experiments are now in progress to test the ability of DLSD and 2-bromo-LSD, but not LLSD, to displace the $[^3H]$-DLSD visualized by autoradiography and thereby identify specifically bound label and possibly the target sites for the 5-HT.

**References**

BENNETT, J.P. & SNYDER, S.H. (1975). Stereospecific binding of D-lysergic acid diethylamide (LSD) to brain membranes: Relationship to serotonin receptors. *Brain Res.*, **94**, 523–544.

CREESE, I., BURT, D. & SNYDER, S.H. (1976). The dopamine receptor: Differential binding of DLSD and related agents of agonist and antagonist states. *Life Sci.*, **17**, 1715–1720.

HOLMAN, R.B. & VOGT, M. (1972). Release of 5-hydroxytryptamine from caudate nucleus and septum. *J. Physiol. (Lond.).*, **223**, 243–254.

LOREZ, H.-P. & RICHARDS, J.G. (1973). Distribution of indolealkylamine nerve terminals in the ventricles of the rat brain. *Z. Zellforsch.*, **144**, 511–522.

LOREZ, H.-P. & RICHARDS, J.G. (1975). 5-HT nerve terminals in the fourth ventricle of the rat brain: Their identification and distribution studied by fluorescence histochemistry and electron microscopy. *Cell. Tiss. Res.*, **165**, 37–48.

RICHARDS, J.G., LOREZ, H.-P. & TRANZER, J.P. (1973). Indolealkylamine nerve terminals in cerebral ventricles: Identification by electron microscopy and fluorescence histochemistry. *Brain Res.*, **57**, 277–288.

RICHARDS, J.G. & TRANZER, J.P. (1974). Ultrastructural evidence for the localization of an indolealkylamine in supra-ependymal nerves from combined cytochemistry and pharmacology. *Experientia*, **30**, 287–289.

---

## 5-HT and LSD high affinity binding sites to brain synaptosomal membranes

G. FILLION, M.P. FILLION, J. JACOB & J.C. ROUSSELLE

*Department of Pharmacology, Pasteur Institute, Paris, France*

5-HT and LSD binding have been studied, using purified synaptosomal membranes isolated from different regions of bovine brain by a density gradient centrifugation technique or in some experiments a lysed $P_2$ fraction isolated either from bovine or from rat brains.

Membranes were incubated at various temperatures (generally 22°C or for different purposes 0°C) in Tris-HCl buffer 50 mM pH 7.4 using tritiated ligands ($[^3H]$-5-HT 17 Ci/mM, $[^3H]$-LSD 22 Ci/mM). Separation of bound and free radioactivity was performed using an ultra-filtration technique (Whatman GFB glass fibre filter) under vacuum. The filter was rinsed with 10 ml of Tris-HCl buffer 0°C and the radioactivity trapped onto the filter was counted by liquid scintillation using Triton X 100 with a Toluene PPO-POPOP mixture.

Previously we described a saturable, reversible, high affinity binding for 5-HT, specific for tryptamines and related structures (Fillion, Fillion, Spinakis, Bahers & Jacob, 1976). Here, parallel studies for 5-HT and LSD bindings show that LSD binding corresponds to a saturable reversible high affinity site with a corresponding dissociation constant similar to that of 5-

HT ($K_D = 2 \times 10^{-9}$M) and a second, saturable and reversible site of less affinity with a corresponding $K_D$ close to 2 to 3 × $10^{-8}$M. Various plotting systems of the binding curve indicate for the second site a positive cooperativity, the Hill coefficient being 3.7. Dissociation rates are quite high for both ligands, however it is much higher for 5-HT than for LSD; both are temperature-dependent with a respective $Q_{10}$ close to 2.5 and 3. Regional distributions of binding capacities for LSD and 5-HT are very similar. They are not homogenous within the brain but vary according to the studied region, e.g. in decreasing order: striatum, hippocampus, cortex, raphé, cerebellum.

Specific lesions of the tryptaminergic system have been performed by stereotaxic injections of 5–6 dihydroxytryptamine within raphe and anterior ventricles; their efficacy has been controlled by inhibition of the uptake of 5-HT. In these conditions, lesions do not modify significantly binding of one or the other ligand; this might indicate a postsynaptic location of the corresponding site.

Comparative assays of the specificity of the high affinity sites for 5-HT and LSD indicate they are related to the tryptaminergic structure but some differences are observed, i.e. bromlysergamide and cyproheptadine are more efficient in displacing LSD than 5-HT (respective $ID_{50}$s are $3 \times 10^{-8}$ M and $6 \times 10^{-7}$M for LSD, $1.5 \times 10^{-6}$ and $6 \times 10^{-6}$ for 5-HT).

Detailed studies of interactions between 5-HT and LSD on these high affinity sites and preliminary assays involving various pretreatments of the membranes might indicate that the different sites observed correspond to an agonist and an antagonist conformation of the same 5-HT receptor-site.

**Reference**

FILLION, G., FILLION, M.P., SPIRAKIS, Ch., BAHERS, J.M. & JACOB, J. (1976). 5-Hydroxytryptamine binding to synaptic membranes from rat brain. *Life Sci.*, **18**, 65–74.

---

# Catecholamine-stimulated prostaglandin synthesis in rat brain synaptosomes

K. HILLIER, P.J. ROBERTS &
P.M. WOOLLARD

*Department of Physiology and Biochemistry and Department of Clinical Pharmacology, University of Southampton SO9 3TU*

Although prostaglandins (PGs) have been shown to be synthesized in brain homogenates from a number of species, little information is available concerning the subcellular distribution and activity of the relevant enzymes (Raffel, Clarenbach, Peskar & Hertting, 1976). It has been suggested that $PGE_2$ may function as a modulator of synaptic transmission in the periphery, where prostaglandin inhibits noradrenaline (NA) release from sympathetic structures (Hedqvist, 1976); it was therefore of interest to find a facilitation of [$^3$H]-NA release from rat brain synaptosomes in the presence of low concentrations of $PGE_2$ (Roberts & Hillier, 1976).

Since the signal for PG release in the peripheral sympathetic system is considered to be associated with the postsynaptic actions of noradrenaline, in this study we have investigated the effects of several

**Table 1** Stimulation of PGE synthesis in rat brain synaptosomes

| *Treatment* | | *Control (ng PGE)* | *Treated (ng PGE)* | *% stimulation* |
|---|---|---|---|---|
| Noradrenaline | 100 µM (6) | 2.41 ± 0.37 | 6.3 ± 0.84* | 161 |
| Dopamine | 100 µM (6) | 2.52 ± 0.37 | 5.14 ± 0.82† | 103 |
| Adrenaline | 100 µM (3) | 1.93 ± 0.14 | 4.7 ± 0.44* | 143 |
| Acetylcholine | 100 µM | | | |
| +physostigmine | 10 µM (2) | 2.1 | 2.2 | — |
| 5-HT | 100 µM (2) | 2.1 | 1.65 | — |
| K+ | 50 mM (5) | 1.94 ± 0.28 | 1.72 ± 0.18 | — |

Results are expressed as means ± s.e. with the number of experiments in parentheses. Levels of significance refer to differences from the results of the control group. * $P < 0.01$; † $P < 0.02$.

neurotransmitter candidates on the PG synthesizing ability of synaptosomes.

Twenty per cent (w/v) homogenates of rat brain tissue (excluding cerebellum) were prepared in 0.32 M sucrose, and crude synaptosomes ($P_2$ fraction) prepared as described by Whittaker (1969). An aliquot (200 µl) of the synaptosome suspension (containing approx. 4 mg protein) was added to 1.3 ml Krebs bicarbonate medium (pH 7.4) containing ascorbic acid (1 mg/ml), isoniazid (10 µM) and, where appropriate, the substance under investigation (Table 1). Synaptosomes were incubated for 5 min at 37°C under 95% $O_2$ : 5% $CO_2$ followed by rapid centrifugation. To each supernatant was added marker amounts (2–5 pg) of [$^3$H]-PGE$_2$ or [$^3$H]-PGF$_2$ and the PG's released into the incubation media assayed, using a modification of the method of Hillier & Dilley (1974). Briefly, this involves selective extraction, column chromatography separation of PGE and PGF groups and radioimmunoassay using antibodies with relatively good selectivity for the PG's under investigation.

The results show that 100 µM amounts of NA, dopamine and adrenaline signficantly stimulate the generation of PGE by synaptosomal tissue. In another experiment NA also significantly stimulated PGF synthesis.

The effect is selective as acetylcholine, 5-HT and $K^+$ depolarization were without a stimulatory effect in this system.

We conclude that (a) contrary to the findings of Raffel *et al.* (1976) noradrenaline and dopamine do stimulate prostaglandin formation in rat brain synaptosomes, (b) this strengthens the link for PGEs exerting a positive feedback on NA release on central nerve terminals as described by Roberts & Hillier (1976).

We thank Dr J. Pike, The Upjohn Co. for the gift of prostaglandins.

**References**

HEDQVIST, P. (1976). Effects of prostaglandins on autonomic neurotransmission. In *Prostaglandins: Physiological, Pharmacological and Pathological Aspects*, ed. Karim, S.M.M., pp. 37–62. Lancaster: M.T.P.

HILLIER, K. & DILLEY, S.R. (1974). Separation and radioimmunoassay of F $_{2\alpha}$ prostaglandins using silica gel microcolumns. *Prostaglandins*, 5, 137–150.

RAFFEL, G., CLARENBACH, P., PESKAR, A. & HERTTING, G. (1976). Synthesis and release of prostaglandins by rat brain synaptosomal fractions. *J. Neurochem.*, **26**, 493–498.

ROBERTS, P.J. & HILLIER, K. (1976). Facilitation of noradrenaline release from rat brain synaptosomes by prostaglandin $E_2$. *Brain Res.* (in press).

WHITTAKER, V.P. (1969). The subcellular fractionation of nervous tissue. In *The Structure and Function of Nervous Tissue*, ed. Bourne, G.H., Vol. 3, pp. 1–24. New York: Academic Press.

---

## Ontogenesis of muscarinic receptor sites in rat brain

C.R. HILEY

*Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX*

Cholinergic neurotransmission in the central nervous system does not appear to be fully functional until the second week of life. Thus rats do not respond to injections of scopolamine with changes in locomotor activity until after the 15th day of life (Campbell, Lytle & Fibiger, 1969) and whilst scopolamine has a synergistic effect on amphetamine-induced gnawing in 30-day-old rats, this synergism could not be seen in 10-day-old rats (McGeer, Fibiger & Wickson, 1971). Both acetylcholinesterase and choline acetyltransferase (ChAc) are present in the rat neostriatum 4 days after birth although ChAc activity is very low (McGeer *et al.*, 1971; Guyenet, Beaujouan & Glowinski, 1975). However, it is possible that the lack of response to scopolamine indicates the absence of the muscarinic cholinoceptor on the postsynaptic neuron rather than the immaturity of the presynaptic pathway. This possibility has been examined by studying the binding of [$^3$H]-atropine to homogenates of neonatal rat cerebral cortex and caudate nucleus.

Homogenates of cerebral cortex and caudate nucleus in Krebs-Henseleit solution were preincubated for 15 min at 37°C before the addition of [$^3$H]-atropine (Radiochemical Centre, Amersham; 245 mCi mmol$^{-1}$). After a further 15 min the incubation was terminated by centrifugation at 14,000 *g* for 30 seconds. The surface of the pellet was rinsed twice with Krebs-Henseleit solution and radioactivity in the pellet was determined by liquid scintillation counting. Two series of samples were incubated together and one of these series contained 10 µM propylbenzilylcholine, a potent antimuscarinic compound, throughout the experiment. Receptor-specific binding of [$^3$H]-atropine was taken to be that fraction of binding which was abolished by the presence of the propylbenzilylcholine.

Receptor-specific binding of $[^3H]$-atropine was present in both the caudate nucleus and cerebral cortex of 5-day-old rats in concentrations which were 20–30% of the adult values. The concentrations increased with age and by 16 days had reached approximately 60% of the adult concentrations of receptor sites which were attained by the fifth week of life. Throughout, the caudate nucleus contained a higher concentration of receptor sites than the cerebral cortex.

These results indicate that the lack of response to scopolamine in young rats is not a consequence of the absence of the muscarinic cholinergic receptor though it is possible that the receptor is not functionally connected to the processes which are responsible for mediating the changes in neuronal activity produced by cholinergic agents.

I am indebted to Caroline Middleton for skilful technical assistance.

**References**

CAMPBELL, B.A., LYTLE, L.D. & FIBIGER, H.C. (1969). Ontogeny of adrenergic arousal and cholinergic inhibitory mechanisms in the rat. *Science*, **166**, 635–637.

GUYENET, P.G., BEAUJOUAN, J.C. & GLOWINSKI, J. (1975). Ontogenesis of neostriatal cholinergic neurones in the rat and development of their sensitivity to neuroleptic drugs. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **288**, 329–334.

McGEER, E.G., FIBIGER, H.C. & WICKSON, V. (1971). Differential development of caudate enzymes in the neonatal rat. *Brain Res.*, **32**, 433–440.

---

## Analysis of end-plate current fluctuations produced by acetylcholine and acetylmonoethylcholine in rat muscle

D. COLQUHOUN

*Department of Pharmacology, St. George's Hospital Medical School, Blackshaw Road, London SW17 0QT*

The characteristics of the ion channels that are opened by agonists in the frog muscle end-plate have been studied by analysis of the fluctuations about the mean end-plate voltage, or current, produced by the drugs (Katz & Miledi, 1972; Anderson & Stevens, 1973; Colquhoun, Dionne, Steinbach & Stevens, 1975). Similar experiments have now been conducted on rat diaphragm muscle fibres (Sakmann, 1976).

Strips of rat diaphragm were bathed in Krebs' solution containing tetrodotoxin (300 nM), at temperatures between 13° and 25°C. End-plate currents were recorded by a voltage clamp method. Analysis of current fluctuations was carried out on 10 s samples, filtered below 0.5 Hz and above 500 Hz. Digital samples were taken at 1 ms intervals. Agonists were applied by perfusion from a wide tipped micropipette (Cooke & Quastel, 1973) so as to produce end-plate currents of 10–90 nA.

The decay of spontaneous miniature end-plate currents (MEPC) showed no consistent deviation from a single exponential; and, equivalently, spectral density curves for both acetylcholine (ACh), and for acetylmonoethylcholine (AMECh) showed only slight deviation from a single Lorentzian form, so a single time constant could be inferred. The time constants for both MEPC decay ($\tau_{\mathrm{MEPC}}$) and for current fluctuations ($\tau_{\mathrm{ACh}}$ or $\tau_{\mathrm{AMECh}}$) decreased with temperature ($Q_{10} = 3.26 \pm 0.63$), and increased as the membrane was hyperpolarized ($109 \pm 7$ mV for an e-fold change in $\tau$). These values are similar to those for frog muscle.

At 20°C, and −80 mV membrane potential, $\tau_{\mathrm{MEPC}}$ was 1.6 ms. This appeared to be slightly (20–50%) slower than $\tau_{\mathrm{ACh}}$, though the size of the discrepancy depended on the method of curve fitting used for spectra. The simplest, but not the only, explanation for such a discrepancy would be that the acetylcholine concentration in the synaptic cleft did not fall rapidly compared with the lifetime of an open channel.

The ratio $\tau_{\mathrm{AMECh}}/\tau_{\mathrm{ACh}}$ was $0.56 \pm 0.05$ (15); this can be interpreted as meaning that the average lifetime of an open ion channel, when the channel is opened by AMECh, is about 56% of its mean lifetime when opened by ACh. This ratio is similar to the ratio of decay time constants for normal (ACh) and 'false' (AMECh) MEPCs observed by Large & Rang (1976).

The mean conductance ($\gamma$) of a single open ion channel, may, under certain assumptions, be inferred from the variance of current fluctuations. No evidence was found for a difference in conductance between channels opened by ACh and AMECh; the values were $\gamma_{\mathrm{ACh}} = 24.9 \pm 1.2$ pS ($n = 36$) (very similar to the value reported for frog muscle by Colquhoun *et al.*, 1975), and $\gamma_{\mathrm{AMECh}} = 26.7 \pm 1.4$ pS ($n = 16$).

I am indebted to Dr Colin Mercer, Institute of Sound and Vibration Research, Southampton University, for data analysis facilities.

**References**

ANDERSON, C.R. & STEVENS, C.F. (1973). Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol. (Lond.)*, **235**, 655–691.

COLQUHOUN, D., DIONNE, V.E., STEINBACH, J.H. & STEVENS, C.F. (1975). Conductance of channels opened by acetylcholine-like drugs in muscle end-plate. *Nature (Lond.)*, **253**, 204–206.

COOKE, J.D. & QUASTEL, D.M.J. (1973). Transmitter release by mammalian motor nerve terminals in response to focal polarization. *J. Physiol. (Lond.)*, **228**, 377–405.

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol. (Lond.)*, **224**, 665–699.

LARGE, W.A. & RANG, H.P. (1976). Analysis of the action of a false transmitter at the neuromuscular junction. *J. Physiol. Proceedings*, **258**, 105–106P.

SAKMANN, B. (1976). Noise analysis of acetylcholine induced currents in normal and denervated rat muscle fibres. *Pflügers Arch.*, **354**, R178.

---

## Pharmacological agents and acute experimental hyperlactataemia in the dog

A.L. LOUBATIÈRES, G. RIBES &
G. VALETTE

*Laboratory of Pharmacology and Pharmacodynamics, Faculty of Medicine of Montpellier, France*

Experimental hyperlactataemia was produced in dogs anaesthetized with pentobarbital 30 mg/kg i.v.: (a) by administration of high doses of biguanides (phenformin 30 mg/kg intraduodenally, metformin 150 mg/kg intraduodenally) (Loubatières, Ribes & Blayac, 1973); (b) by bilateral repeated and prolonged electrical stimulation of the sciatic nerves (unidirectional and rectangular pulses of 10V, 5 ms and 60 Hz) producing repeated and prolonged contractions (60 min) of the gastrocnemius and soleus groups of muscles; (c) by hypoxia following closed circuit inhalation of a mixture of oxygen (9%) and nitrogen (91%) and (d) by continuous i.v. injection of adrenaline (1.5 $\mu$g $\text{kg}^{-1}$ $\text{min}^{-1}$).

An autoanalyser was used to make continuous measurement of blood sugar in haemolysed blood with potassium ferricyanide and plasma lactate with an enzymatic method (Hohorst, 1963; Minaire, Studievic & Foucherand, 1965). Blood lactate and pyruvate were estimated in fractioned samples by the enzymatic method described by Czok & Lamprecht (1970) and blood pH was measured with a KCl electrode.

Insulin, when administered i.v. (0.085 u $\text{kg}^{-1}$ $\text{h}^{-1}$) or produced *endogenously* by i.v. injection of the hypoglycaemic sulfonamide, glibenclamide (0.05 mg/kg) (Loubatières & Mariani, 1967) prevented, delayed or reduced the hyperlactataemia, hyperpyruvicaemia and acidosis which normally followed the administration of biguanides (Loubatières, Ribes & Blayac, 1973). Co-carboxylase (5 mg $\text{kg}^{-1}$ $\text{h}^{-1}$) produced the same effect (Valette, Ribes, Rondot, Loubatières-Mariani & Loubatières, 1975).

The administration of sodium dichloroacetate (30 mg $\text{kg}^{-1}$ $\text{h}^{-1}$) produced a reduction in the hyperlactataemia produced by phenformin (from $50.6 \pm 10.2$ mg/100 ml to $14.3 \pm 2.8$ mg/100 ml, $n = 13$), by intense muscular work (from $42.3 \pm 10.3$ mg/100 ml to $9.0 \pm 1.5$ mg/100 ml, $n = 3$), or by adrenaline (from $61.3 \pm 13$ mg/100 ml to $29 \pm 2.3$ mg/100 ml, $n = 3$). On the other hand, dichloroacetate did not reduce the hyperlactataemia produced by hypoxia (from $84.7 \pm 12.1$ mg/100 ml to $100.8 \pm 8.7$ ml, $n = 3$). These measurements of lactate were made in total blood.

Dichloroacetate slightly reduced the increase in lactataemia which can be observed in the dog presenting a permanent diabetes following the injection of alloxan (50 mg/kg i.v.). Glycaemia was not notably modified.

The therapeutic implications of these observations are under study.

**References**

CZOK, R. & LAMPRECHT, W. (1970). In *Methods of Enzymatic Analysis*, ed. Bergmeyer, H.U., II, 1407, Academic Press: New York.

HOHORST, H.J. (1963). In *Methods of Enzymatic Analysis*, ed. Bergmeyer, H.U. pp. 266–270. Academic Press, New York.

LOUBATIÈRES, A. & MARIANI, M.M. (1967). Etude pharmacologique et pharmacodynamique d'un sulfonylurée hypoglycémiant particulierement actif, le glybenzcylamide. *C.R. Acad. Sc., Paris*, Série D, **267**, 643–645.

LOUBATIÈRES, A., RIBES, G. & BLAYAC, J.P. (1973). L'insuline exogene et endogène s'opposent à l'hyperlactatémie produite chez le chien par l'administration de phenformine, *C.R. Acad. Sc., Paris*, Serie D, **276**, 847–851.

MINAIRE, Y., STUDIEVIC, C. & FOUCHERAND, F. (1965). Dosage automatique de l'acide lactique. Possibilité d'utilisation en continu. *Path. et Biol.*, **13**, 1170–1173.

VALETTE, G., RIBES, G., RONDOT, A.M., LOUBATIÈRES-MARIANI, M.M. & LOUBATIÈRES, A. (1975). Effet de la cocarboxylase sur l'hyperlactatémie et l'hyperpyruvicémie provoquées par la phenformine chez le chien, *C.R. Soc. Biol.*, **169**, 121–125.

## Long-term effects of propranolol on tyrosine hydroxylase and dopamine-β-hydroxylase in the superior cervical ganglia of the rabbit

I.W. CHUBB & A.E.G. RAINE
(introduced by E.M. VAUGHAN WILLIAMS)

*University Department of Pharmacology, South Parks Road, Oxford*

The beta-adrenoceptor antagonist propranolol is well-established as an effective antihypertensive in man, but the mechanism of its hypotensive action has not been fully explained. Experimental studies have established that the immediate effects of propranolol treatment include a reduction in cardiac output and in plasma renin activity. However, this cannot explain the therapeutic effect since the hypotensive action of propranolol is delayed in onset (Prichard & Gillam, 1969). The present study was designed to test the hypothesis that chronic propranolol treatment causes a slow-onset reduction in sympathetic nervous system activity. One reflection of changes in the activity of sympathetic neurons is now considered to be the concentration of tyrosine hydroxylase and dopamine-β-hydroxylase contained within the cells (Thoenen, 1972). We have estimated the amount of these two enzymes in the superior cervical ganglia of rabbits after different lengths of treatment with propranolol.

Eighteen rabbits weighing 2–3 kg were injected subcutaneously twice daily with either saline (controls, 1 mg/kg of a 0.9% w/v NaCl solution, $n=6$) or (±)-propranolol (4 mg/kg for 3 days, $n=4$; 6 days, $n=4$ or 12 days, $n=4$). Fifteen hours after cessation of treatment the left and right superior cervical ganglia were removed and homogenized separately in 1 ml of 0.3M sucrose. Tyrosine hydroxylase activity was determined using the method of Levitt, Gibb, Daly, Lipton & Udenfriend (1967) and dopamine-β-hydroxylase (DBH) activity by that of Molinoff, Weinshilboum & Axelrod (1971). The results are summarized in Table 1.

Prolonged β-adrenoceptor blockade with propranolol induced a gradual reduction in activity of tyrosine hydroxylase. A delayed fall in DBH activity followed, highly significant after 12 days of treatment. These results can be interpreted as indicating a decreased activity of the sympathetic nervous system in response to this drug treatment. This suggestion is supported by the observations of Lewis & Hausler (1975) that propranolol infusion decreases preganglionic sympathetic activity.

Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of noradrenaline (Levitt *et al.*, 1965), and DBH activity has been used as an estimate of the size of the noradrenergic vesicle pool (De Potter & Chubb, 1971). Therefore, it is suggested that the gradual decrease in the concentration of these two enzymes indicates a decreased release of noradrenaline in response to chronic β-adrenoceptor blockade. This could explain the gradual decrease in blood pressure which is a well-established effect of treatment with propranolol.

### References

DE POTTER, W.P. & CHUBB, I.W. (1971). The turnover rate of noradrenergic vesicles. *Biochem. J.*, **125**, 375–376.

LEVITT, M., SPECTOR, S., SJOERDSMA, A. & UDENFRIEND, S. (1965). Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharm. exp. Ther.*, **148**, 1–8.

LEVITT, M., GIBB, J.W., DALY, J.W., LIPTON, M. & UDENFRIEND, S. (1967). A new class of tyrosine hydroxylase inhibitors and a simple assay of inhibition *in vivo*. *Biochem. Pharmacol.*, **16**, 1313–1321.

LEWIS, P.J. & HAUSLER, G. (1975). Reduction in sympathetic nervous activity as a mechanism for hypotensive effect of propranolol. *Nature*, **256**, 440.

MOLINOFF, P.B., WEINSHILBOUM, R. & AXELROD, J. (1971). A sensitive enzymatic assay for dopamine-β-hydroxylase. *J. Pharm. exp. Ther.*, **178**, 425–431.

PRICHARD, B.N.C. & GILLAM, P.M.S. (1969). Treatment of hypertension with propranolol. *Br. med. J.*, **1**, 7–16.

THOENEN, H. (1972). Neuronally mediated enzyme induction in adrenergic neurons and adrenal chromaffin cells. *Biochem. Soc. Symp.*, **36**, 3–15.

**Table 1** Tyrosine hydroxylase and dopamine β-hydroxylase (DBH) activities of rabbit superior cervical ganglia (SCG)

| | | *SCG tyrosine hydroxylase activity (nmol 15 min$^{-1}$ mg protein$^{-1}$)* | *SCG DBH activity (nmol h$^{-1}$ mg protein$^{-1}$)* |
|---|---|---|---|
| Controls | ($n=12$) | 1.74 ± 0.12 | 185 ± 5 |
| Propranolol 4 mg/kg. bd. | 3 days ($n=8$) | 1.62 ± 0.14 | 196 ± 7 |
| Propranolol | 6 days ($n=8$) | 1.29 ± 0.11† | 152 ± 10* |
| Propranolol | 12 days ($n=8$) | 1.34 ± 0.10* | 139 ± 8‡ |

* $P<0.05$; † $P<0.02$; ‡ $P<0.001$.

# Action of cytochrome C on transmembrane potentials of normal or hypoxic guinea-pig myocardial strips

M.C. AUCLAIR, P. JOURDON, P. LECHAT & M. LEMEIGNAN

*Institut de Pharmacologie, LA. 206 C.N.R.S., 21, rue de l'Ecole de Médecine, 75006 Paris, France*

It has been previously reported that cytochrome C modified the cardiac membrane effects produced by hypoxia (Lechat, Auclair, Dechezleprêtre & Lemeignan, 1975). The mechanisms involved were investigated on isolated stimulated (1 Hz) right ventricle strips of guinea-pig heart. The strips were placed in Tyrode solution saturated with either 95% $O_2$ and 5% $CO_2$ (normal strips) or 95% $N_2$ and 5% $CO_2$ (hypoxic strips). Action potentials (A.P.) were recorded using microelectrodes and contractions using a transducer.

In normal strips cytochrome C (1 µg/ml) did not modify either the A.P. or the contraction [number of experiments ($n$) = 35]. In hypoxic strips cytochrome C did not modify the resting potentials. The plateau phase and the A.P. duration decreased under hypoxic conditions ($n=30$). Addition of cytochrome C again increased them ($n=20$), but the contraction which had also decreased under hypoxic conditions was not restored. After blocking the sodium-calcium channel by $MnCl_2$ (10 mM), the action of hypoxia became more rapid ($n=8$), but cytochrome C failed to induce the previous effects ($n=8$). In $K^+$ and $Ca^{2+}$ rich Tyrode solution ($K^+ \times 10$; $Ca^{2+} \times 4$) with an equimolar reduction in $Na^+$, the rapid sodium channel was blocked and slow A.P. could be induced by stimulation, which disappeared under hypoxic conditions. Addition of cytochrome C delayed their disappearance (75 min instead of 35 min) but did not increase the amplitude and duration of the slow A.P. These results showed that cytochrome C did not modify the rapid sodium movements, but was acting by interfering with the calcium-sodium movements. However, a direct activation of the slow calcium-sodium current, isoprenaline-like, is probably not involved since cytochrome C did not modify the slow A.P. configuration. In order to see if, in cytochrome C effects, an eventual modification of $K^+$ movements was implied, the action of tetraethylammonium (TEA) was studied in similar conditions. As with cytochrome C, TEA again increased the A.P. duration which had decreased under hypoxia, as well as the plateau phase. Contrary to cytochrome C, TEA lengthened the A.P. repolarization of normal strips ($n=6$), did not maintain the slow A.P. and restored the contraction which had decreased under hypoxic conditions ($n=6$).

Cytochrome C could maintain, in hypoxia, the slow inward calcium-sodium movements of the guinea-pig myocardial membrane, being however unable to restore contraction. The mechanism of this action could be at least partly related to a change in $K^+$ movements, without excluding other pathways.

**Reference**

LECHAT, P., AUCLAIR, M.C., DECHEZLEPRÊTRE, S. & LEMEIGNAN, M. (1975). Effects of cytochrome C on functional consequences of different types of experimental hypoxia. *6th Int. Congress Pharmacology, Abstracts*, 545, p. 236.

---

# Analysis of the effects of isoxsuprine on guinea-pig atria and trachea

D.G. FARRAR & ANNE VERITY

*Department of Pharmacology and Biochemistry, Research and Development Laboratories, Fisons Ltd, Pharmaceutical Division, Loughborough, Leics*

Isoxsuprine is a smooth muscle relaxant used in the treatment of cerebral and peripheral vascular disorders and in premature labour. Initial studies suggested that it possessed $\beta$-adrenoceptor stimulant effects, a direct papaverine-like action and $\alpha$-adrenoceptor blocking activity on various smooth muscle preparations (Lish, Dungan & Peters, 1960). In the present study, the effects of isoxsuprine on the guinea-pig trachea (Coleman & Farmer, 1971) and spontaneously beating paired guinea-pig atria have been examined.

On the trachea, isoxsuprine (0.03–3.0 µg/ml) added cumulatively, caused a dose-dependent relaxation ($ED_{50} = 0.12 \pm 0.03$ µg/ml; $n=12$). Isoxsuprine was 120 times less potent than isoprenaline and gave a similar maximal response. The response was inhibited by propranolol (10 ng/ml, dose ratio $= 1136 \pm 282$: $n=8$). This marked inhibition of isoxsuprine was greater than that observed with standard $\beta$-adrenoceptor stimulants. For example, the dose-ratio obtained with salbutamol was $12.3 \pm 4.1$ ($n=4$). Cocaine (10 µg/ml) had no effect on responses to isoxsuprine. The response to a submaximal dose of isoxsuprine (1 µg/ml) was not subject to tachyphylaxis on repeated administration. The effects of larger doses of isoxsuprine, however, were not repeatable. For example isoxsuprine (10 µg/ml) given as a single dose.

only caused 69.1 ± 6.2% relaxation ($n=6$). A further relaxation could not be obtained by increasing the dose of isoxsuprine into the range 20–80 µg/ml, but instead a contraction was obtained. Doses of isoxsuprine greater than 80 µg/ml, however, did relax the preparation. In the presence of isoxsuprine (10 µg/ml) the sensitivity of the trachea to isoprenaline was reduced.

On spontaneously beating atria (bathed in McEwens solution at 37°C) isoxsuprine (0.1–10 µg/ml) increased both the rate and force of contraction. The peak change in rate was only 45.7 ± 6.8% ($n=4$) of that achieved with isoprenaline. Responses to isoxsuprine (10 µg/ml) were completely abolished by propranolol (10 ng/ml, $n=4$), but were not affected by cocaine (3 µg/ml, $n=4$). The sensitivity of the atria to isoprenaline was reduced after the application of isoxsuprine. Decreases in the rate and force of contraction were often obtained with large doses of isoxsuprine (> 10 µg/ml), particularly when administered in the presence of propranolol.

It is concluded that responses of the guinea-pig atria and trachea to low concentrations of isoxsuprine (up to 10 µg/ml) are mediated by $\beta$-adrenoceptors. The results suggested that isoxsuprine had partial agonist properties on both preparations. Despite structural similarities to tyramine and other indirectly-acting sympathomimetics, the effects of isoxsuprine did not include an indirect sympathomimetic component. There was evidence that at high concentrations isoxsuprine possessed non-specific depressant activity. The pharmacological profile of isoxsuprine on these tissues is very similar to that described for the structural analogue, oxyfedrine (Sakai, Shiraki & Hashimoto, 1973).

**References**

COLEMAN, R.A. & FARMER, J.B. (1971). The inducement of tone and its inhibition in isolated tracheal muscle. *J. Pharm. Pharmac.*, **23**, 220–222.

LISH, P.M., DUNGAN, K.W. & PETERS, E.L. (1960). A survey of the effects of isoxsuprine on non-vascular smooth muscle. *J. Pharmac. exp. Ther.*, **129**, 191–199.

SAKAI, K., SHIRAKI, Y. & HASHIMOTO, K. (1973). An analysis of the $\beta$-adrenergic action of oxyfedrine. *Eur. J. Pharmac.*, **21**, 81–88.

---

# Altered reactivity of stomach fundus smooth muscle in Okamoto spontaneous hypertension

J. ALTMAN, F. Da PONTE & M. WORCEL

*Physiologie & Pharmacologie, INSERM U7, Hôpital Necker, 75015 Paris, France*

Okamoto & Aoki (1963) obtained a strain of spontaneously hypertensive rats (SHR) which develop a disease that resembles human essential hypertension. Shibata & Kurahashi (1972), Bohr (1974) and Janis & Triggle (1973) have observed that the response of SHR carotid and aortic muscle to $Sr^{2+}$, $La^{3+}$, $Mn^{2+}$, $Ba^{2+}$, $Ca^{2+}$ and diazoxide is changed in SHR, suggesting an alteration in excitation-contraction coupling mechanisms in SHR arterial smooth muscle. In order to know if the changes in reactivity are related to the primary cause of hypertension in SHR or are just an adaptation induced by the high arterial blood pressure (Folkow, Hallbäck, Lundgren, Sivertsson & Weiss, 1972) we have proceeded to test the contractile response of a visceral smooth muscle. A longitudinal strip of the rat fundus of 20 weeks old male and female SHR and Wistar normotensive (NW) rats was prepared according to the method of Vane (1957). All the following experiments were performed in a normal physiological Tris buffered solution at 37°C and gassed with 100% $O_2$. After equilibration during 2 h the contractile response to $SrCl_2$, $MnCl_2$, $LaCl_3$ and $BaCl_2$ was examined. The response to $Ba^{2+}$ in male SHR strips was lower ($ED_{50}$ $1.4 \times 10^{-3} \pm 0.3$ M, $n=10$) than in the male NW strips ($3.0 \times 10^{-4} \pm 0.2$ M, $n=10$; $P<0.01$). Maximal responses were identical. Male SHR fundus strips contracted much more with $Sr^{2+}$ (SHR: 42 ± 3% of Mx response to $Ba^{2+}$, $n+10$; NW: 19 ± 4, $n=10$, $P<0.01$) than NW strips. This confirms Bohr's (1974) results but on the other hand there was no difference in the response to both $BaCl_2$ and $SrCl_2$ between female SHR and NW fundus strips, and $MnCl_2$ and $LaCl_3$ were relaxant in all cases. The dose-response curves to $Ca^{2+}$ of depolarized SHR and NW fundus strips and the study of the antagonism of diazoxide on $Ca^{2+}$ contractions was performed using Janis & Triggle (1973) method. The contractile action of $Ca^{2+}$ in depolarized preparation was enhanced in both male and female SHR strips. The effect of diazoxide was more marked in SHR strips than in NW fundus strips. In conclusion, SHR fundus smooth muscle presents the same modification of reactivity to $Ba^{2+}$, $Sr^{2+}$, $Ca^{2+}$ and diazoxide that was previously described in arterial smooth muscle. This indicates that the cellular modification responsible for the increase of vascular tonus in SHR is not an adaptable reaction to high blood pressure. The differences between female SHR and male SHR responses are not unexpected considering the natural evolution of hypertension in Okamoto rats which is milder in female SHR.

This work was supported by grants of the I.N.S.E.R.M. and D.G.R.S.T., France.

**References**

BOHR, D.F. (1974). Reactivity of vascular smooth muscle from normal and hypertensive rats: effect of several cations. *Fed. Proc.*, **33**, 127–132.

FOLKOW, B., HALLBÄCK, M., LUNDGREN, Y., SIVERTSSON, R. & WEISS, L. (1972). The importance of adaptative changes in vascular design for the establishment and maintenance of primary hypertension, as studied in man and in spontaneously hypertensive rats. In *Spontaneous Hypertension*, ed. K. Okamoto, pp. 103–114. Heidelberg: Springer-Verlag.

JANIS, R.A. & TRIGGLE, D.J. (1973). Effect of diazoxide on aortic reactivity to calcium in spontaneously hypertensive rats. *Can. J. Physiol. Pharmacol.*, **51**, 621–626.

OKAMOTO, K. & AOKI, K. (1963). Development of a strain of spontaneously hypertensive rats. *Jap. Circulation J.*, **27**, 282–293.

SHIBATA, S. & KURAHASHI, K. (1972). Possible mechanisms of vascular reactivity differences in spontaneously hypertensive and normotensive rat aortae. In *Spontaneous Hypertension*, ed. K. Okamoto, pp. 115–118. Heidelberg: Springer-Verlag.

VANE, J.R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Brit. J. Pharmacol.*, **12**, 244–349.

---

# Molecular mechanism of post-nephrectomy uterine supersensitivity to angiotensin

E. CHEVILLOTTE, M.A. DEVYNCK, P. MEYER,
A. PAPADIMITRIOU, B. ROUZAIRE-DUBOIS & M. WORCEL

*Physiologie et Pharmacologie, INSERM U7, Hôpital Necker, 75015 Paris, France*

Bilateral nephrectomy has been shown to induce a specific supersensitivity of the response of isolated rat uteri to angiotensin II which is expressed by a shift to the left of the dose-response curve. This phenomenon could not be explained by a simple freeing of receptor sites secondary to the disappearance of endogenous angiotensin. The $pA_2$ values of two competitive antagonists of angiotensin II were not modified by nephrectomy suggesting that supersensitivity was related neither to a change in the affinity of receptors nor to a variation of a possible equilibrium existing between active and inactive receptor conformations (Meyer, Papadimitriou & Worcel, 1974). If angiotensin receptor affinity is unchanged after nephrectomy, two hypotheses may explain the supersensitivity to angiotensin, both based on the obligatory assumption that a limiting factor of the angiotensin effect after nephrectomy exists beyond the receptor site (i) *increase in the number of receptors.* If the limiting factor is distal to the receptor site, an increase in the number of receptors would be responsible for the supersensitivity, and maximal contraction would be obtained at an angiotensin concentration lower than in control experiments, (ii) *no variation in the number of receptors but an increase in the 'efficiency' of the limiting factor distal to receptors.* In this case, an increased response would result from the same number of hormone-receptor interactions.

In order to test these hypotheses, we have studied the specific binding of a [$^3$H]-angiotensin II with high specific activity and intact biological activity to membranes of uteri isolated from normal and nephrectomized rats. In normal rats, the specific binding of [$^3$H]-angiotensin II to uterine membranes was half saturated and saturated at angiotensin concentrations of $2 \times 10^{-8}$ M and $10^{-7}$ M respectively. The similarity of these values to the $ED_{50}$ value and to the angiotensin concentration inducing maximal contraction suggests that in normal rats a linear relation exists between angiotensin receptor occupancy and contraction.

Bilateral nephrectomy produces, after 18–20 h, a 70% increase in the total number of binding sites (control 0.7 pmol mg$^{-1}$; nephrectomized 1.2 pmol mg$^{-1}$ protein) which cannot be accounted for by variations in the occupancy of receptor sites, and no significant variation in the apparent dissociation constant (Chevillotte, Rouzaire-Dubois, Devynck & Meyer, 1974). It has also been demonstrated that the variations in angiotensin receptor number was directly related to plasma angiotensin levels.

These pharmacological and biochemical results allow the following conclusions which will be analysed in this communication: (i) Supersensitivity of uterine muscle to angiotensin observed after nephrectomy is secondary to a true increase in angiotensin receptors without significant variation in their affinity for the hormone, (ii) the association of an increase in angiotensin receptors and a shift to the left of the dose-response curve implies the existence of limiting factor(s) distal to the receptor, (iii) the consequence of the existence of this limiting factor is that after nephrectomy, some receptors behave as 'spare receptors', (iv) no evidence for the existence of spare receptors could be demonstrated in normal uteri.

**References**

MEYER, P., PAPADIMITRIOU, A. & WORCEL, M. (1974). Specific supersensitivity of smooth muscle to angiotensin II after nephrectomy. *Br. J. Pharmac.*, **52**, 435–440.

CHEVILLOTTE, E., ROUZAIRE-DUBOIS, B., DEVYNCK, M.A. & MEYER, P. (1974). Variations in the number of uterine angiotensin receptors following changes in plasma angiotensin levels. *Eur. J. Pharmacol.* (in press).

---

# Evolution of aortic and cardiac cyclic AMP phosphodiesterase during the onset of mineralocorticoid hypertension in the rat

C. LUGNIER, T. MICHON & J.C. STOCLET

*Laboratoire de Pharmacodynamie, Faculté de Pharmacie, Université Louis Pasteur, 3 rue de l'Argonne, 67083 Strasbourg, France*

Amer (1975) suggested that increased activity of cyclic AMP phosphodiesterase (PDE) in heart and aorta may play a part in the onset of hypertension in the rat. The present investigation was undertaken to determine whether the increase of PDE activity precedes or follows the rise of BP during mineralocorticoid hypertension in the rat. Male Sprague Dawley rats were implanted with 100 mg desoxycorticosterone acetate in 4 pellets and subsequently were given *ad libitum* a 9% w/v NaCl solution. The rats were killed by decapitation. The heart and the aorta (freed from adventitia layer) were rapidly removed and homogenized as described previously (Lugnier & Stoclet, 1974). PDE activity was determined in crude homogenates using a method modified from Thompson & Appleman (1971). The modification consisted of measuring the yield of the separation of the reaction products from residual $^3$H-cyclic AMP by adding [$^{14}$C]-adenosine to each tube and of using a different resin for the separation (QAE Sephadex A25). Protein was measured according to Lowry, Rosenbrough, Farr & Randall (1951).

Table 1 shows that cardiac PDE measured at both substrate concentrations increased with age in control but not in hypertensive rats, where it was already maximal after 2 weeks of treatment. In the aorta the only observed modification was an increase of PDE measured at low substrate concentration in hypertensive rats, after 2 weeks of treatment.

The data show that modifications of cardiac and aortic PDE specific activity occur during the onset of mineralocorticoid hypertension but are no more apparent during the chronic phase of hypertension.

**Table 1** Variations of the specific activity of cyclic AMP phosphodiesterase (PDE) with age and hypertension, in heart and aorta from control and mineralocorticoid hypertensive rats

| | *Systolic BP* | *PDE (pmol . min$^{-1}$ . mg protein$^{-1}$) (2)* | | | |
|---|---|---|---|---|---|
| | | *Heart* | | *Aorta* | |
| | *(mmHg) (1)* | *$1 \times 10^{-4}$ M (3)* | *$1 \times 10^{-6}$ M (3)* | *$1 \times 10^{-4}$ M (3)* | *$1 \times 10^{-6}$ M (3)* |
| | | *8 weeks old (after 2 weeks of treatment)* | | | |
| Control | 115±2 | 474.7±12.6 | 74.5±1.0 | 1143.2±157.8 | 174.0±10.7 |
| Hypertensive | 163±2† | 1447.0±80.0† | 165.0±3.0† | 1385.4±124.0 | 246.0±4.5† |
| | | *16 weeks old (after 10 weeks of treatment)* | | | |
| Control | 146±3* | 1188.6±43.9* | 164.1±4.2* | 1575.8±185.0 | 186.2±7.5 |
| Hypertensive | 184±9† | 1274.2±55.0 | 163.8±1.6 | 1595.4±135.0 | 192.5±6.9 |

(1) Mean of 10 rats ± s.e. mean.
(2) Mean of 4 determinations on pools 10 organs, ± s.e. mean.
(3) Substrate concentration.
Student's *t* tests: † $P < 0.001$ compared with controls.
* $P < 0.001$ compared with 8 weeks old rats.

This investigation was supported by grants from D.G.R.S.T. and I.N.S.E.R.M.

**References**

AMER, M.S. (1975). Cyclic nucleotides in disease; on the biochemical etiology of hypertension. *Life Sci.*, **17**, 1021–1038.

LOWRY, D.H., ROSENBROUGH, N.J., FARR, R.L. & RANDALL, R.J. (1951). Protein measurement with the Folin reagent. *J. biol. Chem.*, **193**, 265–275.

LUGNIER, C. & STOCLET, J.C. (1974). Inhibition by papaverine of cGMP and cAMP phosphodiesterases from the rat heart. *Biochem. Pharmacol.*, **23**, 3071–3074.

THOMPSON, W.J. & APPLEMAN, M.M. (1971). Multiple cyclic phosphodiesterase activities from rat brain. *Biochemistry*, **10**, 311–316.

---

## Evidence of central cardiovascular effects of intracerebroventricular isoprenaline in anaesthetized rat

Y. COHEN, A. LINDENBAUM, D. PORQUET & J. WEPIERRE

*Laboratoire de Pharmacodynamie, Centre d'Etudes pharmaceutiques, 92290, Chatenay Malabry, France*

Isoprenaline (1, 2 and 4 µg) injected intracerebroventricularly in urethane anaesthetized rat produced a long lasting hypotension and tachycardia. It is unlikely that these effects are related to leakage in peripheral circulation of the amine because: (1) After intraventricular injection, $[^3H]$-isoprenaline diffused partially out of the central nervous system, but maximal blood and heart levels measured 5 min after administration were about 2 ng/g. These concentrations were unable to induce cardiovascular effects when injected intravenously. (2) In rats cephalic cross-circulation experiments indicated that intraventricular injection of 8 µg isoprenaline to the rat donor produced tachycardia which was not observed in the second animal.

The present study showed that isoprenaline had mainly central cardiovascular effects after intracerebroventricular injection.

---

## Characteristics and altered sensitivity of cerebral $\beta$-adrenoceptors assessed by $[^3H]$-propranolol binding

S.R. NAHORSKI & BRENDA M. SMITH

*Department of Pharmacology and Therapeutics, School of Medicine, University of Leicester, Leicester, LE1 7RH, and Department of Pharmacology, University of Sheffield, Sheffield, S10 2TN*

Studies on the nature and characteristics of the $\beta$-adrenoceptor have been greatly assisted by the observation that in many tissues this receptor is closely associated with the enzyme adenylate cyclase (Robison, Butcher & Sutherland, 1971). We have previously utilized this approach in assessing catecholamine-induced cyclic AMP formation in chick cerebral hemispheres and have provided evidence that these effects are mediated by a $\beta$adrenoceptor (Nahorski, Rogers, Smith & Anson, 1975). In the present study we have extended our experiments on the characterization of this receptor by examining the binding of $^3H$ propranolol, a specific ligand for the $\beta$-adrenoceptor (Nahorski, 1976), to chick cerebral membranes.

Experiments were performed on 1–6 day old male Ranger chicks. Cyclic AMP formation was determined in 0.37 mm incubated slices of the cerebral hemispheres by a protein binding assay. $[^3H]$ (±)-propranolol binding was examined in a crude synaptic membrane fraction prepared by differential centrifugation (Nahorski, 1976). The order of potency of the catecholamines to stimulate cyclic AMP formation, isoprenaline > adrenaline > noradrenaline, was also observed in the ability of these compounds to displace $[^3H]$-propranolol from membrane binding sites. Salbutamol, although only a partial agonist, had a similar potency to adrenaline in both of these systems and dopamine was inactive at concentrations up to 100 µM.

(−)-Propranolol was a potent antagonist of isoprenaline (1 µM)-stimulated cyclic AMP formation ($IC_{50} = 7 \times 10^{-8}$ M) and $[^3H]$-propranolol binding ($IC_{50} = 1 \times 10^{-8}$M). (+)-Propranolol was about 100-fold less potent in both systems. H35/25(1-(*p*-tolyl)-2-isopropylamino-1-propanol), a relatively specific $\beta_2$-

adrenoceptor antagonist (Carlsson, Ablat, Brandström & Carlsson, 1972), was about 300–500-fold more potent than the cardioselective $\beta_1$-adrenoceptor antagonists, metoprolol and practolol in its ability to antagonize isoprenaline-stimulated cyclic AMP formation and [$^3$H]-propranolol binding.

In previous experiments we have demonstrated that chronic depletion of cerebral catecholamines results in an increased responsiveness of $\beta$-adrenoceptor mediated cyclic AMP formation whereas chronic isoprenaline administration induces a loss of sensitivity of this response (Nahorski & Rogers, 1975). In order to assess the possibility of an altered affinity and/or number of cerebral receptor sites in these conditions, the binding of [$^3$H]-propranolol was examined in cerebral membranes of these animals. The affinity and total number of [$^3$H]-propranolol binding sites were identical in reserpine (3 × 2.5 mg/kg) and vehicle-treated chicks. However, following isoprenaline treatment (2 × 150 μmol/kg) the loss of $\beta$-adrenoceptor responsiveness was accompanied by a significant (25–30%) apparent loss of total binding sites.

The experiments described suggest that the $\beta$-adrenoceptor in chick cerebral hemispheres resembles that found in bronchial and vascular smooth muscle ($\beta_2$) rather than that in heart ($\beta_1$). In addition evidence is presented to suggest that the loss of responsiveness of cerebral $\beta$-adrenoceptors following chronic exposure to isoprenaline is associated with a loss of available receptor binding sites.

**References**

CARLSSON, E., ABLAT, B., BRANDSTRÖM, A. & CARLSSON, B. (1972). Differentiated blockade of chronotropic effects of different adrenergic stimulants in cat heart. *Life Sci.*, **11**, 953–958.

NAHORSKI, S.R. (1976). Association of high affinity stereospecific binding of $^3$H-propranolol to cerebral membranes with $\beta$-adrenoceptors. *Nature (Lond.)*, **259**, 488–489.

NAHORSKI, S.R. & ROGERS, K.J. (1975). Altered sensitivity of $\beta$-adrenoceptor-mediated cyclic AMP formation in brain. *Br. J. Pharmac.*, **55**, 300–301P.

NAHORSKI, S.R., ROGERS, K.J., SMITH, B.M. & ANSON, J. (1975). Characterisation of the adrenoceptor mediating changes in cyclic adenosine 3′-5′-monophosphate in chick cerebral hemispheres. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **291**, 101–110.

ROBISON, G.A., BUTCHER, R.W. & SUTHERLAND, E.W. (1971). Cyclic AMP. New York: Academic Press.

---

# Peripheral effects of the amphetamine-type anorectic drugs: inhibition of catecholamine-induced lipolysis, respiration, glucose utilization in the adipose tissue of man and rat

M. DUBOST, A. ESCOUSSE, J. MOUNIÉ & R. TRUCHOT

*Laboratory of Medical Pharmacology, University of Dijon*

Some phenylethylamine derivatives are among the most important anorectic drugs which act by a central mechanism, but their peripheral metabolic actions are poorly understood.

It appears that many of the amphetamine drugs are weak agonists of the lipolysis in adipose cells but an inhibitory effect of sympathomimetically-induced lipolysis was found in the case of fenfluramine. The main body of work reported in this field has been carried out in the rat, the adipose adrenoceptor of which may well be quite different from that in man.

For this reason it seemed essential to investigate the effects of amphetamine anorectic drugs in both species (rat and man) on: (a) The adrenoceptor agonist activity for lipolysis, respiration, glucose oxidation of the

**Table 1**

| *Products* | *Species* | *Lipolysis with theophylline* | *Lipolysis with theophylline + NA* | *Respiration* |
|---|---|---|---|---|
| Fenfluramine | Man | ↓ | ↓ | ↑ |
| | Rat | no effect | ↓ | no effect |
| Fenproporex | Man | ↓ | ↓ | ↓ |
| | Rat | ↓ | ↓ | ↓ |
| Chlorphentermine | Man | no effect | ↓ | ↓ |
| | Rat | ↓ | ↓ | ↓ |

adipocytes, and (b) the antagonist effect on metabolic peripheral actions of catecholamines (lipolytic, and calorigenic actions).

Experiments were performed *in vitro* using the following products: fenfluramine (2-ethylamino-1-(3-trifluoro-methylphenyl)propane); fenproporex (+)-1-(methyl-1-phenyl-2-ethyl amino)3-propionitrile), chlorphentermine (dimethyl-1,1-chlorophenyl-2-ethylamine).

Oxygen uptake, lipolysis (estimated as glycerol release in incubation medium) and glucose utilization, were measured in the Warburg apparatus with Krebs Ringer bicarbonate solutions containing noradrenaline (NA) ($8 \times 10^{-5}$ M) or theophylline ($1 \times 10^{-5}$ M) or NA + theophylline + anorectic drug ($1 \times 10^{-4}$ to $1.4 \times 10^{-3}$M) on epididymal fat pads of 200 g Wistar rats. In man studies were conducted in epiploic (or perirenal) adipose tissue obtained during appendicectomy of 40 subjects of both sexes (17 to 72 years of age). Results are given in Table 1.

---

## Activity of anorectic drugs (amphetamine), amfepramone and UP 507–04) on two models of obesity in animals

G. DUMEUR, BERNADETTE HÜE, J.M. LWOFF, M.A. MOURIES & D. TREMBLAY

*Research Department, UPSA Laboratories, 128, rue Danton, 92504 Rueil Malmaison, France*

The anorectic activity of three drugs (amphetamine, amfepramone and UP 507–04 (cyclopropyl-2 p-chlorophenyl-4 methyl-5 pyrrolidine succinate)) was studied on two models of experimental obesity: gold thioglucose treated mice and rats after bilateral electrolytic lesions of hypothalamic ventromedian nucleus.

The obesity, on these models, developed in two phases: dynamic and static. During the dynamic phase the rate of weight gain was very pronounced, during the static phase very poor. In plasma, total lipids, triglycerides and cholesterol levels were increased in obese rats.

Mice and rats were treated orally by drugs for 12 days during the static phase. Amphetamine (4 mg/kg in rats and mice) induced a sharp decrease of food intake and body weight without statistically significant modifications of total lipids, triglycerides and cholesterol in rats. Amfepramone (16 mg/kg) in mice had no activity on food intake and body weight. UP 507–04 (8 mg/kg in mice and 4 mg/kg in rats) decreased food intake and body weight; total lipids, triglycerides and cholesterol tended to remain at normal values in rats.

---

## Decremental skin conductance response in mice, during iterative photostimulation; an attention-sustaining capacity model for psychopharmacological research

R. MARCY & M.A. QUERMONNE

*Department of Pharmacology, University of Caen Pharmaceutical Unit, 14032 Caen Cedex, France*

As a result of repeated presentation of a stimulus to which attention is attracted, a decrement and eventual extinction of the skin conductance reaction (SCR) occurs. Delivering iterative photostimulation to mice while recording their palmar SCRs, Marcy, Quermonne & Nammathao (1976) have demonstrated that SCR extinction (i.e. habituation) is delayed by psychoanaleptics.

In this study, the same method was used. Time of extinction (in 100ths of an h) was computed against dose (mg/kg) for each drug tested. Depending on the effect obtained, the following parameters were determined: standard delaying dose, i.e. delaying SCR extinction until 125 (SDD) or standard shortening dose, i.e. speeding up SCR extinction to time 50 (SSD).

Unlike the psychoanaleptics tested, central depressants speeded up SCR extinction time. Clonazepam was more active than phenobarbitone (cf. lower SSD in Table 1) although it is definitely less active in suppressing the righting reflex. Delay of habituation obtained with amphetamines was confirmed with related compounds. Rather unexpectedly, both fenfluramine, although considered a depressant, and, above all, piracetam, although devoid of any stimulant property, also delayed SCR extinction. In fact, fenfluramine possesses some stimulant properties and piracetam improves learning

**Table 1** Effect of drugs on extinction of skin conductance reaction

| *Drugs* | *No. b* | *Dose-range*<br>*Delaying effect* | *SDD*<br>*(mg/kg i.p.)* | *analysis of linear regression variance*<br>*V* | <br>*F* | <br>*P* |
|---|---|---|---|---|---|---|
| Dexamphetamine<br>(*sulphate, sal.*) | 7 | 0.0312–8.00 | 0.0722<br>(±0.015) | 347081.31 | 896.93 | <0.001 |
| Piracetam (*sal.*) | 4 | 0.50–10.00 | 0.787<br>(±0.146) | 164007.61 | 197.40 | <0.001 |
| Dexamphetamine<br>(*tartrate, c.m.c.*) | 4 | 1.00–5.00 | 1.40<br>(±0.111) | 123764.97 | 525.16 | <0.001 |
| Methamphetamine<br>(*sal.*) | 3 | 2.00–3.00 | 2.01<br>(±0.040) | 45212.47 | 211.21 | <0.001 |
| Phentermine<br>(*sal.*) | 3 | 5.00–20.00 | 4.84<br>(±0.522) | 17964.87 | 73.48 | <0.001 |
| Fenfluramine<br>(HCl, sal.) | 4 | 5.00–30.00 | 10.06<br>(±1.036) | 35616.91 | 185.43 | <0.001 |
| Benzphetamine<br>(HCl, sal.) | 3 | 12.50–30.00 | 29.17<br>(±1.954) | 13353.17 | 67.81 | <0.001 |
| Ephedrine<br>(HCl, sal.) | 3 | 20.00–60.00 | 34.68<br>(±3.086) | 15674.47 | 83.63 | <0.001 |
| Caffeine<br>(*+benzoate Na,*<br>*dist. water*) | 4 | 80.00–150.00 | 150.50<br>(±5.162) | 12176.72 | 143.89 | <0.001 |
| Pyrisuccideanol<br>(*dimaleate, sal.*) | 4 | 250.00–1000.00 | 956.80<br>(±104.29) | 8126.07 | 48.00 | <0.001 |
|  | *Speeding up effect* |  | *SSD*<br>*(mg/kg i.p.)* |  |  |  |
| Clonazepam<br>(*c.m.c.*) | 7 | 0.156–10.00 | 1.00<br>(±0.19) | 20688.50 | 132.82 | <0.001 |
| Phenobarbital<br>(*Na, sal.*) | 4 | 10.00–35.00 | 22.41<br>(±1.76) | 12524.41 | 88.69 | <0.001 |

*No b.* = number of batches of 10 mice for the relevant experiment.
SDD, SSD = see text. Figures in brackets are confidence limits $P < 0.05$.
$V$ = variance, $F$ = variance ratio, $P$ = probability.
sal. = dissolved in saline, c.m.c. = suspended in 0.5% carboxymethylcellulose water solution.

in particular conditions (Sara & David-Remacle, 1974).

Decremental SCR during monotonous solicitation may be proposed as a model for attention sustaining capacity under the effect of drugs. Results obtained show that this model is not exclusively influenced by drugs modifying general activity.

Conversely, it seems particularly sensitive to specific central effects which are not readily demonstrable otherwise.

**References**

MARCY, R., QUERMONNE, M.A. & NAMMATHAO, B. (1976). Habituation to iterative photostimulation in the palmar skin conductance response of mice, its delay by psychoanaleptics. *Experientia (Basel)*, **32**, 208–209.

SARA, S.J. & DAVID-REMACLE, M. (1974). Recovery from electroconvulsive shock-induced amnesia by exposure to the training environment: pharmacological enhancement by piracetam. *Psychopharmacologia (Berl.)*, **36**, 59–66.

## Effect of the 1,5-benzodiazepines, clobazam and triflubazam, on the sleep of man

CORAL H. CLARKE, A.N. NICHOLSON & BARBARA M. STONE

*Royal Air Force Institute of Aviation Medicine, Farnborough, Hampshire*

Many of the 1,4-benzodiazepines used as hypnotics have persistent effects on performance, but it would appear that the 1,5-benzodiazepine, clobazam, has limited residual sequelae (Caille & Bassano, 1974; Borland & Nicholson, 1975). The possibility that 1,5-benzodiazepines may possess less potential for impairing performance in man raises the question of their use as hypnotics for persons involved in skilled activity the next day. It is in this context that we have investigated the hypnotic properties of two members of this group of drugs – clobazam (1-methyl-5-phenyl-7-chloro-1H-1, 5-benzodiazepine-2, 4(3H, 5H)-dione) and triflubazam (1-methyl-5-phenyl-7-trifluoromethyl-1H-1, 5-benzodiazepine-2, 4(3H, 5H)-dione).

The subjects were six healthy male volunteers aged between 21 and 28 years. The assessment of each treatment (matching placebos or dose or a drug) involved four days. For two nights the subjects slept at home and retired at a set time, and for the next two nights they slept in the laboratory. They were requested to refrain from napping and undue exercise, and to abstain from caffeine and alcohol from mid-day on the days which involved recordings. Nine to twelve days separated each assessment. On each occasion the subject ingested two identical capsules, and the study was double blind. Preliminary studies suggested that the appropriate dose ranges to study were clobazam (10–20 mg) and triflubazam (20–40 mg).

The effect of the drugs was limited to the night of ingestion. With both drugs no statistically significant changes were observed in total sleep time or in the duration (min) of each sleep stage. With clobazam (10 & 20 mg) sleep onset latency (SOL) was reduced ($P<0.05$), and the latency to stage 3 was shortened. There was some evidence of reduced awakenings to stage 0 and stage 1 activity, and with both 10 and clobazam (20 mg) the percentage of total sleep time occupied by stage 1 activity was less ($P<0.01$). With clobazam (20 mg) there was an increase in the percentage of stage 2 sleep ($P<0.05$) related to the second two hourly interval of sleep from SOL, and an overall decrease in the percentage of stage 3 ($P<0.01$) and stage $3+4$ ($P<0.05$) sleep. No changes were observed in rapid eye movement sleep.

With triflubazam (20 & 40 mg) there was no change in latencies to sleep stages. There was some evidence of reduced awakenings to stage 0 and stage 1 activity. With 20 mg the percentage of total sleep time occupied by stage 1 activity was less ($P<0.05$), but this change was not observed with the 40 mg dose. No other changes were observed.

It would appear that the hypnotic activity of the 1,5-benzodiazepines is limited, and that this is particularly so in the case of triflubazam. However, the effect of clobazam on sleep, and its restricted effects on performance (Borland & Nicholson, 1975) suggest that it may be a useful drug in the management of limited sleep difficulties in persons involved in skilled activity.

### References

BORLAND, R.G. & NICHOLSON, A.N. (1975). Immediate effects on human performance of a 1,5-benzodiazepine (clobazam) compared with the 1,4-benzodiazepines, chlordiazepoxide hydrochloride and diazepam. *Br. J. clin. Pharmac.*, **2**, 215–221.

CAILLE, E.J. & BASSANO, J.-L. (1974). Étude de quatre benzodiazépines au niveau de l'électrogenèse cérébrale, du sommeil, du comportement et de l'humeur. *J. Pharmacol., Clin.*, **4**, 241–255.

---

## Behavioural changes in rats suggesting drug-induced headache

A.P. SILVERMAN

*ICI Ltd., Central Toxicology Laboratory*

Nitroglycerin and similar organic nitrates lower blood-pressure and are liable to induce headache in man after inhalation or skin contact. If rats also get headaches, they would be expected to show: (1) Conditioned taste aversion; drugs like lithium producing unpleasant symptoms in man often cause rats to avoid novel but otherwise attractive bait (Nachman & Hartely, 1975). (2) An unspecific reduction in spontaneous behaviour (whereas strongly conditioned behaviour, e.g. to avoid shock, might overcome a mild deficit).

Twenty-four albino rats were caged in pairs, separated for 6 h each week day, and given water for 2 h daily while separated. Social behaviour was observed on return to the home cage (Silverman,

1971) classifying the acts and postures described by Grant & Mackintosh (1963) as non-social (exploration, grooming, etc.), social approach (including aggression), and escape from the other rat.

Nitroglycerin was administered as a 10% solution in corn-oil, syringing 0.5 ml/kg on to the shaved flank (Clark & Litchfield, 1967) 30 min after removing water and 15–30 min before test. Treating only one rat of each pair, nitroglycerin did not affect social behaviour. When both were treated, the total number of actions of all kinds observed in 3 min averaged 262 (±21 s.e. mean) for the six pairs of nitroglycerin-treated rats. This was a decline of 20 ± 20 actions from the pre-experimental baseline, while corn-oil controls increased by 48 ± 26 to 302 ± 24. The difference was as predicted ($t = 2.04$, $P < 0.05$ 1-tailed) but was mainly in social approach, with little change in non-social or escape behaviour.

Conditioned aversion: Instead of water, rats were offered a solution of 0.1% saccharin sodium on the day nitroglycerin was first administered. Comparing water on the previous day with saccharine on day 2 (21 h after dosing), controls increased intake by 10.2 ± 1.4 g, rats given nitroglycerin by only 7.1 ± 1.6 g ($t = 1.81$, $df = 20$, $P < 0.05$ 1-tailed).

Tolerance has been reported to nitroglycerin with supersensitivity following withdrawal, and another conditioned aversion experiment tested this.

On days 1–5 rats were offered 0.1% saccharin for 1 h in the morning, treated with nitroglycerin (50 mg/kg) or ethylene glycol dinitrate (EGDN) or corn-oil ($n = 10$) and later offered water for 1 hour. Groups differed little in intake of either fluid, and were left untreated on days 6 and 7. On day 8 they were offered saccharine given a novel flavour with coffee and dosed again. On day 9, corn-oil alone increased coffee intake by 6.1 ± 2.5 g, EGDN by 0.1 ± 1.5 g and nitroglycerin reduced it by 0.9 ± 2.1 g ($F_{2,27} = 3.39$, $P < 0.05$).

Effects in both social behaviour and conditioned aversion tests were in the predicted direction but were much smaller than expected, perhaps because the high solubility of nitroglycerin in corn-oil reduced the effective dose.

I thank Dr G. McCallum (Nobel's Explosives Co.) for nitroglycerin and EGDN, and Miss Ayleen Hughes for technical assistance.

### References

CLARK, D.G. & LITCHFIELD, M.H. (1967). Metabolism of ethylene glycol dinitrate and its influence on blood-pressure of the rat. *Br. J. Ind. Med.*, **24**, 320–325.

GRANT, E.C. & MACKINTOSH, J.H. (1963). A comparison of the social postures of some common laboratory rodents. *Behaviour*, 21, 246–259.

NACHMAN, M. & HARTLEY, P.H. (1975). Illness and learned taste aversion. *J. comp. physiol. Psychol.*, **89**, 1010–1018.

SILVERMAN, A.P. (1971). Behaviour of rats given a 'smoking dose' of nicotine. *Anim. Behav.*, **19**, 67–74.

---

## The release of endogenous amino acids from the cat spinal cord *in vivo*

G.E. FAGG, C.C. JORDAN &
R.A. WEBSTER

*Department of Pharmacology, University College London, Gower Street, London WC1E 6BT*

Perfusion of the central canal of the cat spinal cord *in vivo* is a useful approach to the study of spinal neurotransmission (Morton, Stagg & Webster, 1976). Employing this technique, Jordan & Webster (1971) demonstrated the release of acetylcholine and radiolabelled glycine into the perfusate (in the presence of eserine and *p*-hydroxymercuribenzoate, respectively) during stimulation of femoral and sciatic nerves.

In the present experiments, cats were spinalized at C1 under halothane anaesthesia, immobilized with gallamine triethiodide and the central canal (L4–S1) was perfused (0.06 ml/min) with artificial CSF (Feldberg & Fleischhauer, 1960). A bipolar platinum electrode (tip separation 5 mm) was inserted into the cord at C2 to deliver trains (0.1 Hz, 5 s duration) of stimuli (12V, 2 ms, 40 Hz) for 10 min periods. Samples of perfusate were collected (10 min periods) and assayed for amino acids by a modification of the dansylation procedure described by Briel & Neuhoff (1972), utilizing [$^3$H]-dansyl chloride with [$^{14}$C]-leucine as an internal standard.

Table 1 shows those amino acids studied. Introduction of *p*-chloromercuriphenylsulphonate (pCMS) $10^{-4}$ M into the artificial CSF, in an attempt to inhibit amino acid uptake (Balcar & Johnston, 1973), significantly increased the efflux of several amino acids and further significant increases resulted from stimulation of descending spinal tracts. Stimulation in the absence of pCMS did not consistently increase the efflux of any amino acid studied (values not shown). The stimulated increases in glutamate, glycine, GABA and alanine efflux could be related to their proposed neurotransmitter roles (Curtis & Johnston, 1974) although the evoked release

**Table 1** Release of endogenous amino acids from the cat spinal cord *in vivo*

| *Amino acid* | *Release following:* | |
|---|---|---|
| | *pCMS $10^{-4}$ M (% spontaneous)* | *pCMS $10^{-4}$ M + stimulation (% pCMS $10^{-4}$ M release)* |
| | *A* | *B* |
| Leucine | 186 ± 26 (10)† | 137 ± 11 (4)‡ |
| Alanine | 233 ± 57 (7) | 229 ± 67 (4)‡ |
| Aspartate | 179 ± 30 (8)* | 264 ± 102 (3) |
| GABA | 387 ± 98 (10)* | 163 ± 11 (4)§ |
| Glutamate | 197 ± 25 (8)† | 186 ± 13 (3)‡ |
| Glutamine | 100 ± 6 (10) | Not calculated |
| Glycine | 850 ± 142 (10)† | 194 ± 41 (4)‡ |
| Lysine | 72 ± 8 (5)* | 130 ± 21 (3) |
| Proline | 256 ± 60 (6)* | 169 ± 29 (4) |

Spontaneous release of amino acids was determined in the absence of pCMS. The $^3$H radioactivity in each dansyl derivative was corrected for recovery by means of a [$^{14}$C] leucine internal standard and values were normalized with respect to the spontaneous efflux of endogenous leucine.

*A*: Release after 60 min perfusion with pCMS $10^{-4}$ M in artificial CSF. Values are per cent of spontaneous release.

*B*: Maximum release following stimulation in the presence of pCMS $10^{-4}$ M. Values are per cent of pCMS $10^{-4}$M release.

All values are mean ± s.e. mean (number of observations in parentheses). Significance levels (2-tail paired *t*-test): $P<0.05$* or 0.01† compared with spontaneous release. $P<0.05$‡ or 0.01§ compared with release following pCMS $10^{-4}$ M. For all other comparisons $P>0.1$.

of leucine (not a neurotransmitter candidate) indicates that caution is necessary in this interpretation.

The valuable assistance provided by I.M. Jones is much appreciated. G.E.F. is an S.R.C. scholar.

This work was supported in part by an M.R.C. project grant to R.A.W.

#### References

BALCAR, V.J. & JOHNSTON, G.A.R. (1973). High affinity uptake of transmitters: studies on the uptake of L-aspartate, GABA, L-glutamate and glycine in cat spinal cord. *J. Neurochem.*, **20**, 529–539.

BRIEL, G. & NEUHOFF, V. (1972). Microanalysis of amino acids and their determination in biological material using dansyl chloride. *Hoppe-Seyler's Z. physiol. Chem.*, **353**, 540–553.

CURTIS, D.R. & JOHNSTON, G.A.R. (1974). Amino acid transmitters in the mammalian central nervous system. *Ergebn. Physiol.*, **69**, 98–188.

FELDBERG, W. & FLEISCHHAUER, K. (1960). Penetration of bromophenol blue from the perfused cerebral ventricles into the brain tissue. *J. Physiol. (Lond.)*., **150**, 451–462.

JORDAN, C.C. & WEBSTER, R.A. (1971). Release of acetylcholine and [$^{14}$C]-glycine from the cat spinal cord *in vivo*. *Br. J. Pharmac.*, **43**, 441P.

MORTON, I.K.M., STAGG, C.J. & WEBSTER, R.A. (1976). Perfusion of the central canal and subarachnoid space of the cat and rabbit spinal cord *in vivo*. *Neuropharmacology* (In press).

---

## The effect of cortisol on $\alpha_1$-macroglobulin and $\alpha_2$-acute phase globulin in the arthritic rat.

D.A. LEWIS & D.P. PARROTT

*Department of Pharmacy, University of Aston in Birmingham*

Recent work has shown that plasma antiproteases may be anti-inflammatory since they inhibit a number of proteases that are involved in inflammation (Barrett & Starkey, 1973; Hercz, 1974). The two most abundant human plasma antiproteases are $\alpha_1$-antitrypsin and $\alpha_2$-macroglobulin. In rat plasma the corresponding pair of antiproteases are $\alpha_1$-antitrypsin and $\alpha_1$-macroglobulin. A third antiprotease, $\alpha_2$-acute phase globulin, is produced as a result of inflammation. Both $\alpha_1$-macroglobulin and $\alpha_2$-acute phase globulin are similar in properties to human $\alpha_2$-macroglobulin and can be assayed enzymatically by

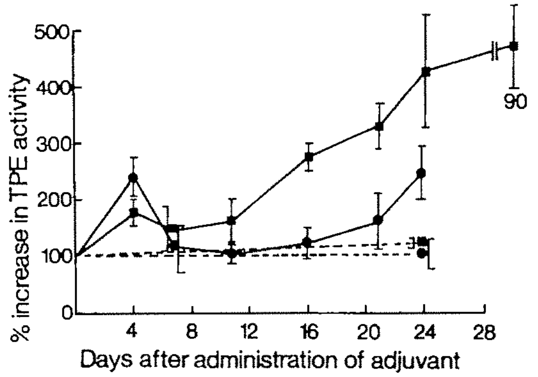


**Figure 1** Trypsin-protein-esterase (TPE) values of the plasma of arthritic rats ■——■ and arthritic rats treated with cortisol ●——●. Dotted lines represent non-arthritic control animals. Each result represents the mean of five animals and vertical lines indicate s.e. mean.

measuring their trypsin-protein esterase (TPE) activity (Ganrot, 1973).

In this communication we report on the action of cortisol on the plasma levels of $\alpha_1$-macroglobulin and $\alpha_2$-acute phase protein in the normal and adjuvant arthritic rat.

Adjuvant arthritis was induced in male, Wistar strain rats (180–200 g body weight) (Parrott & Lewis, 1974). Some normal and some arthritic rats were injected daily, subcutaneously, with cortisol acetate in saline (5 mg/kg body weight) over the experimental period. The controls were injected with saline. $\alpha_1$-macroglobulin and $\alpha_2$-acute phase globulin were assayed simultaneously by measuring the TPE activity of plasma.

The results show that TPE plasma levels were elevated by inflammation but that cortisol treatment depressed these levels towards that of the non-arthritic controls. Cortisol had no significant effect on non-Arthritic rat plasma TPE levels.

Cortisol stimulates $\alpha_1$-antitrypsin levels in the blood of both normal and arthritic rats (Parrott & Lewis, 1976) but clearly it has no effect on $\alpha_1$-macroglobulin or $\alpha_2$-acute phase globulin levels apart from depressing acute phase antiproteases through its anti-inflammatory action.

### References

BARRETT, A.J. & STARKEY, P.M. (1973). The interaction of $\alpha_2$-macroglobulin with proteinases. *Biochem. J.*, **133**, 709–724.

GANROT, K. (1973). Rat $\alpha_2$-acute phase globulin, a human $\alpha_2$-macroglobulin homologue. *Biochim. biophys. Acta.*, **322**, 62–67.

HERCZ, A. (1974). The inhibition of proteinases by human $\alpha_1$-antitrypsin. *Eur. J. Biochem.*, **49**, 287–292.

PARROTT, D.P. & LEWIS, D.A. (1974). Transcortin levels in the blood of arthritic rats. *Biochem. Pharmac.*, **24**, 925–927.

PARROTT, D.P. & LEWIS, D.A. (1976). Protease and antiprotease levels in the blood of arthritic rats. *Ann. rheum. Dis.* (In press).

---

## Potentiation of anaphylactic bronchoconstriction by non-steroidal anti-inflammatory agents

P. MILLER & P. ROBSON
(introduced by G.W.L. JAMES)

*Department of Pharmacology, Roussel Laboratories Ltd., Kingfisher Drive, Covingham, Swindon, Wiltshire*

Non-steroidal anti-inflammatory agents have been reported to enhance mediator release from both human and guinea-pig sensitized lung challenged *in vitro* (Walker, 1972; Engineer, Piper & Sirois, 1976). We have extended these observations to the *in vivo* situation and report here the effect of non-steroidal anti-inflammatory agents on anaphylactic bronchoconstriction in the guinea-pig.

Guinea-pigs were sensitized either passively or actively. Passive sensitization was by intravenous injection of 0.5 ml of a 1/50 dilution of serum prepared according to the method of Davies & Johnston (1971) and were challenged 24 h later. Active sensitization was by the intraperitoneal and subcutaneous injection of ovalbumen (100 mg/kg) with antigen challenge three weeks later. Anaphylactic bronchoconstriction was measured according to the method of Collier & James (1967). Inhibitory compounds and antigen (ovalbumen) were administered intravenously, the compounds being given 5 min before antigen.

Sodium meclofenamate (1 mg/kg) significantly potentiated anaphylactic bronchoconstriction in passively sensitized guinea-pigs when submaximal doses of antigen (0.12–0.60 mg/kg) were used for challenge. When animals were challenged with a maximal dose of antigen (15 mg/kg), sodium meclofenamate had no significant effect. In animals challenged with a maximal dose of antigen in which the histamine-induced component of bronchoconstriction had been suppressed by the administration of mepyramine (2 mg/kg), sodium meclofenamate potentiated the reaction, returning the bronchoconstriction to the level of that seen in control animals.

In animals treated in this way however, mepyramine plus meclofenamate significantly inhibited the reaction over the first minute following antigen challenge, the period of the reaction identified by Collier & James (1967) as being mediated by kinins. Similar results were obtained for flufenamic acid (5 mg/kg).

In actively sensitized guinea-pigs, similar results were obtained, although there were quantitative differences. Thus, mepyramine was less effective in blocking bronchoconstriction following challenge with antigen (0.6 mg/kg of ovalbumen) in active than passively sensitized animals. Moreover, whereas in passively sensitized guinea-pigs mepyramine inhibited over the whole of the 10 min period for which the reaction was followed, in actively sensitized animals mepyramine inhibited the reaction only over the initial 4–5 minutes. Sodium meclofenamate had no effect when given alone but potentiated the reaction in mepyramine treated animals; again, however, the initial period of bronchoconstriction was inhibited in animals given mepyramine plus meclofenamate.

These results confirm the potentiation of mediator release by non-steroidal anti-inflammatory agents and have shown its importance in an *in vivo* situation. From the results obtained, it appears that sodium meclofenamate potentiated the SRS-A mediated portion of anaphylactic bronchoconstriction, probably through an effect upon the synthesis of prostaglandins thought to play a modulatory role in the control of mediator release (Walker, 1972). Differences were observed between the relative importance of the mediators in anaphylactic bronchoconstriction in actively and passively sensitized animals, and it appears that histamine is less important in actively sensitized animals.

**References**

COLLIER, H.O.J. & JAMES, G.W.L. (1967). Humoral factors affecting pulmonary inflation during acute anaphylaxis in the guinea-pig *in vivo*. *Br. J. Pharmac.*, **30**, 283–301.

DAVIES, G.E. & JOHNSTON, T.P. (1971). Quantitative studies on anaphylaxis in guinea-pigs passively sensitized with homologous antibody. *Int. Archs. Allergy appl. Immunol.*, **41**, 648–654.

ENGINEER, D.M., PIPER, P.J. & SIROIS, P. (1976). Interaction between the release of SRS-A and of prostaglandins. *Br. J. Pharmac.*, **57**, 460–461P.

WALKER, J.L. (1972). The regulatory function of prostaglandins in the release of histamine and SRS-A from passively sensitized human lung tissue. In *Advances in the Biosciences*, ed. Bergström, S. & Bernhard, S., pp. 235–240. Braunschweig: Pergamon Press Vieweg.

---

## Effects of activation of $H_1$- and $H_2$-receptors on central cardiovascular structures in cats and on behaviour in chickens

B. DELBARRE, H. SCHMITT & DANIELLE SENON

*Département de Pharmacologie, Faculté de Médecine Broussais, 15, rue de l'Ecole de Médecine, 75270 Paris Cédex 6*

Intracerebroventricular administration of histamine increased blood pressure, heart rate and amplified sympathetic discharges. Intracerebroventricular administrations of mepyramine but not of metiamide have been reported to reduce this effect which was therefore suggested to be mediated by activation of $H_1$-receptors (Finch & Hicks, 1976).

In chloralose-anaesthetized cats, histamine, 4-methylhistamine, 2-(2-aminoethyl) pyridine and betahistine were administered into the lateral ventricle of the brain. Histamine (2–10 µg/kg) induced a rise in blood pressure which was reduced by mepyramine indicating the involvement of $H_1$-receptors. 2-(2 aminoethyl) pyridine, and betahistine, two stimulants of $H_1$-receptors, induced hypertension and tachycardia. The pressor effect reached 35 mmHg after 100 µg/kg of 2-(2 aminoethyl) pyridine and 15 mmHg after 200 µg/kg of betahistine.

These effects were suppressed by an intraventricular administration of mepyramine (100 µg/kg) but not of metiamide (50 µg/kg). High doses of 4-methylhistamine (100 µg/kg), a stimulant of $H_2$-receptors, injected into the lateral ventricle of the brain, induced a brief increase in blood pressure (15–20 mmHg). At higher doses, this effect was followed by a hypotension, possibly due to leakage into the systemic circulation. The rise in blood pressure was abolished by an intracerebroventricular injection of metiamide (100 µg/kg) and of mepyramine (50 µg/kg).

Histamine in newborn chickens induced sleep characterized by a loss of the righting reflex. The duration of sleep was 12 min after 50 mg/kg, i.m. of the drug. 4-Methylhistamine (50 mg/kg, i.m.) induced sleep for 6 min, but 2-(2 aminoethyl) pyridine and betahistine (100 mg/kg, i.m.) failed to abolish the righting reflex.

These experiments show the predominant role of the activation of $H_1$-receptors for the stimulation of central cardiovascular structures, but activation of $H_2$-receptors induced also an effect possibly by increasing the level of 3′5′-cyclic AMP which has been shown to increase blood pressure when introduced into the lateral ventricle of the brain (Delbarre, Senon & Schmitt, 1975). In contrast, $H_2$- but not $H_1$-receptors seem to play a role in inducing sleep in chickens.

**Reference**

DELBARRE, B., SENON, DANIELLE & SCHMITT, H. (1975). Study on the cardiovascular action of dibutyryl-3, 5-cyclic AMP (DB.AMP) following the intraventricular administration to cats. Abstracts Sixth International Congress of Pharmacology, Helsinki, 1975, No. 516, p. 227.

FINCH, L. & HICKS, P.E. (1976). Central hypertensive action of histamine in conscious normotensive cats. *Eur. J. Pharmacol.*, **36**, 263–266.

---

## Histamine receptors in the cranial circulation of the monkey

J.W. DUCKWORTH, J.W. LANCE,
G.D.A. LORD & E.J. MYLECHARANE
(introduced by C. RAPER)

*School of Medicine, University of New South Wales, The Prince Henry Hospital, Sydney, Australia*

Histamine has been implicated in the pathogenesis of cluster headache and migraine, but histamine $H_1$-receptor antagonists such as mepyramine have been of little therapeutic benefit in these disorders (Anthony & Lance, 1971). This may well be due to the nature of the cranial vascular histamine receptors. To investigate the nature of these receptors, we have compared the effects of the $H_2$-receptor antagonists, metiamide (Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973) and cimetidine (Brimblecombe, Duncan, Durant, Ganellin, Parsons & Black, 1975), with those of mepyramine, on cranial and systemic vasodilator responses to histamine in *Macaca nemestrinas* monkeys, anaesthetized with i.v. pentobarbitone sodium.

Common and external carotid blood flows on one side were measured simultaneously using an electromagnetic flowmetering technique. Blood pressure was monitored at the carotid bifurcation. Internal and external carotid vascular resistances were calculated from these measurements. Systemic blood pressure was measured in the aortic arch. Cumulative dose-response curves for the decreases in internal and external carotid vascular resistance produced by intracarotid histamine infusions, and dose-response curves for the systemic vasodepressor effects of i.v. histamine doses, were established. Antagonism was assessed in terms of shifts of dose-response curves, expressed as dose ratios (means and 95% confidence limits).

Metiamide was administered to seven monkeys, in progressively increasing doses of 0.25, 1 and 5 mg/kg at 90 min intervals. External carotid dose-response curves were shifted to the right following the 1 and 5 mg/kg doses, the dose ratios being 1.7(1.1–2.6) and 4.1(2.4–6.9) respectively. The internal carotid dose ratios at these dose levels were 1.3(0.7–2.3) and 4.7(1.0–21.0) respectively. In four monkeys treated similarly with cimetidine, the external carotid dose ratios following the 1 and 5 mg/kg doses were 2.0(1.6–2.5) and 7.2(2.1–24.1) respectively, while the internal carotid dose ratios were 1.0(0.4–2.6) and 2.7(0.7–10.7) respectively. These doses of metiamide and cimetidine had no effect on vasodepressor responses. When mepyramine (2 mg/kg) was subsequently administered to these $H_2$-receptor antagonist-treated monkeys, vasodepressor responses were abolished and internal carotid dose-response curves were shifted further to the right. However, there was no appreciable increase in external carotid blockade.

In four monkeys, mepyramine was administered initially. Progressively increasing doses of 0.5, 2 and 10 mg/kg produced dose-dependent shifts to the right of vasodepressor dose-response curves, the dose ratios being 3.0(0.5–16.7), 6.3(1.1–37.3) and 8.3 range (6.7–10.4). After the 10 mg/kg dose, the internal carotid dose ratio was 2.0(0.1–37.6), and the external carotid dose ratio was only 1.8(0.4–8.2). Subsequent administration of metiamide or cimetidine (1 mg/kg) did not alter mepyramine-induced reduction of vasopressor responses, but increased the effect in both cranial circulations.

These results suggest that the cranial vasodilator effects of histamine are mediated predominantly by $H_2$-receceptors in the external carotid circulation, and by both $H_1$- and $H_2$-receptors in the internal carotid vasculature.

**References**

ANTHONY, M. & LANCE, J.W. (1971). Histamine and serotonin in cluster headache. *Archs. Neurol.*, **25**, 225–231.

BLACK, J.W., DUNCAN, W.A.M., EMMETT, J.C., GANELLIN, C.R., HESSELBO, T., PARSONS, M.E. & WYLLIE, J.H. (1973). Metiamide – an orally active histamine $H_2$-receptor antagonist. *Agents and Actions*, **3**, 133–137.

BRIMBLECOMBE, R.W., DUNCAN, W.A.M., DURANT, G.J., GANELLIN, C.R., PARSONS, M.E. & BLACK, J.W. (1975). The pharmacology of cimetidine, a new histamine $H_2$-receptor antagonist. *Br. J. Pharmac.*, **53**, 435P–436P.

# Dopaminergic (co-mediator) modulation of release of histamine and SRS-A in the calf

J.F. BURKA, P. EYRE &
M.C. HOLROYDE

*Pharmacology Laboratory, Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada*

Mast cells of ruminants contain dopamine (Falk, Nystedt, Rosengren & Stenflo, 1964; Aviado & Sadavongvivad, 1970), which is released from sensitized tissue by antigen (Eyre, 1971a). Lung, especially pulmonary vasculature, is an important anaphylactic shock organ in the calf (Eyre, 1971b; Eyre, Lewis & Wells, 1973), whose pulmonary blood vessels possess dopamine receptors which may participate in anaphylaxis. A role for pulmonary dopamine in cattle has not been established.

During studies of adrenergic modulation of anaphylaxis, it seemed important to investigate possible modulatory effects of dopamine.

Calves were sensitized to horse plasma as previously described (Eyre *et al.*, 1973). Blood was collected and the granulocyte fraction separated (Holrodye & Eyre, 1975). Calves were killed with pentobarbitone and lungs removed and 'chopped' (Eyre, 1971a; Burka & Eyre, 1974). Aliquots of washed granulocytes or chopped lung were incubated at 37°C with antigen in buffered saline. Supernates were assayed for histamine and SRS-A (Eyre, 1971a; Burka & Eyre, 1974). Concentration ranges of dopamine, or dopamine plus antagonist were added to the mixture prior to antigen and incubation. Modulation of histamine or SRS-A release was expressed as a percentage of that released by antigen alone. Appropriate corrections were made for 'spontaneous' mediator release in the absence of antigen.

Dopamine ($10^{-7}$ to $10^{-6}$ M) significantly potentiated antigen-induced release of histamine and SRS-A from lung ($P<0.05$). Dopamine ($10^{-6}$ to $10^{-3}$ M) did not alter histamine release from leucocytes. In the presence of spiperone ($10^{-7}$ to $10^{-5}$ M), the potentiating effect of dopamine ($10^{-6}$ M) on SRS-A release was abolished. By contrast, spiperone ($10^{-7}$ to $10^{-5}$M) did not affect the dopamine-enhanced release of histamine. Phentolamine or propranolol ($10^{-8}$ to $10^{-5}$M) did not antagonize the enhancing properties of dopamine.

Other work in this laboratory has shown unusual adrenergic modulation of SRS-A release from calf lung and of histamine release from leucocytes. In leucocytes, adrenergic modulation is biphasic: a potentiatory phase ($\beta$-adrenoceptor mediated) and an inhibitory phase ($\alpha$-adrenoceptor mediated). Dopaminergic receptors may be absent in leucocytes and dopamine does not stimulate $\alpha$- or $\beta$-adrenoceptors at concentrations tested.

In lung by contrast, our previous work has shown that both $\alpha$- and $\beta$-adrenoceptor stimulation inhibited histamine and SRS-A release from calf lung.

Dopamine consistently *enhances* anaphylactic mediator release and when liberated with other mediators from local mast cells, dopamine may reinforce the release of the other mediators by positive feedback. Dopamine itself has very low activity on pulmonary smooth muscle in calves and therefore should not be described as a true mediator. Dopamine may be regarded as a 'co-mediator' of pulmonary anaphylaxis. As far as can be ascertained this situation has not been described previously.

## References

AVIADO, D.M. & SADAVONGVIVAD, C. (1970). Pharmacological significance of biogenic amines in the lungs: noradrenaline and dopamine. *Br. J. Pharmac.*, **38**, 374–385.

BURKA, J.F. & EYRE, P. (1974). The immunological release of slow-reacting substance of anaphylaxis from bovine lung. *Can. J. Physiol. Pharmac.*, **52**, 1201–1204.

EYRE, P. (1971a). Release of dopamine from bovine lung by specific antigen and compound 48/80. *Br. J. Pharmac.*, **42**, 423–427.

EYRE, P. (1971b). Histamine release from calf lung *in vitro* by specific antigen and by compound 48/80. *Arch. int. Pharmac. Ther.*, **192**, 347–352.

EYRE, P., LEWIS, A.J. & WELLS, P.W. (1973). Acute systemic anaphylaxis in the calf. *Br. J. Pharmac.*, **47**, 504–516.

FALCK, B., NYSTEDT, T., ROSENGREN, E. & STENFLO, J. (1964). Dopamine and mast cells in ruminants. *Acta pharmac. tox.*, **21**, 51–58.

HOLRODYE, M.C. & EYRE, P. (1975). Immunologic release of histamine from bovine polymorphonuclear leukocytes. *Am. J. Vet. Res.*, **36**, 1801–1802.

# Calcium and the inhibition of histamine release from rat peritoneal mast cells by non-steroid anti-inflammatory agents

B.J.R. WHITTLE

*Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN*

Non-steroid anti-inflammatory drugs inhibit the release of histamine from rat peritoneal mast cells induced by a variety of stimulants including antigen and compound 48/80 (Thomas & Whittle, 1976). Although these effects could result from an elevation of mast cell cyclic AMP levels via phosphodiesterase inhibition, histamine release stimulated by the calcium ionophore (A23187) was also inhibited, suggesting that other inhibitory mechanisms may also operate; an increase in cyclic AMP levels is not thought to prevent ionophore-induced histamine release (Garland & Mongar, 1976). In the present study, the possibilities that non-steroid anti-inflammatory drugs affect the mobilization of calcium ions, or the processes of oxidative metabolism involved in histamine release, have been investigated.

Mast cells were obtained by lavage of the rat peritoneal cavity with a modified buffered medium (pH 7) and the histamine release following incubation was determined by fluorometric assay. Submaximal histamine release (50–70% of the total histamine content of the mast cells), induced by compound 48/80 (0.15–0.3 μg/ml) or ionophore-A23187 (0.25–0.5 μg/ml) was inhibited by 2, 4 dinitrophenol (50–100 μg/ml) which uncouples oxidative phosphorylation, and by antimycin-A (0.02–0.5 μg/ml) which acts on the cytochrome system. This inhibition was prevented by preincubation with glucose (1–5 mM)-containing medium (10 experiments), as shown by others (see Diamant, 1975). In contrast, the inhibition of histamine release by indomethacin (10–80 μg/ml) or sodium meclofenamate (0.5–10 μg/ml) was not significantly altered by glucose (1–5 mM)-incubation (8 experiments).

Histamine release induced by the ionophore-A23187 was dependent on the dose (0.1–0.5 μg/ml) and on the calcium concentration of the incubation medium. Optimal histamine release, with a submaximal dose of the ionophore (0.33 μg/ml), was obtained with a calcium concentration of 0.75 mM; an increase in the calcium concentration up to 3 mM did not elevate the release further. The dose-dependent inhibition by indomethacin (10–40 μg/ml) or meclofenamate (1–5 μg/ml) of histamine release stimulated by this ionophore was related to the calcium concentration of the incubation medium. In four experiments, the marked inhibition of ionophore (0.33 μg/ml)-induced release by meclofenamate (5 μg/ml) was significantly reduced (from 81.4 ± 2.9% to 7.6 ± 3.9%, mean ± s.e. mean; $P < 0.001$) when the calcium concentration was increased from 0.75 mM to 3 mM. In contrast, the inhibition of ionophore (0.33 μg/ml)-induced release by antimycin-A was not reversed by an increase in the calcium concentration (5 experiments).

These results show that whereas the inhibition of histamine release from rat mast cells, by drugs known to interfere with energy production from oxidative metabolism, can be reversed by incubation with glucose (by promoting anaerobic glycolysis), glucose-incubation did not prevent the inhibition of release produced by indomethacin or meclofenamate. The observation that an increase in the calcium concentration abolished the inhibition of ionophore-stimulated release suggests an action on a calcium-dependent stage in the release process. It has been shown that several aspirin-like drugs can interfere with the uptake and binding of calcium in various tissues (Northover, 1973). Thus, the inhibition of histamine release by non-steroid anti-inflammatory drugs could reflect actions on calcium influx into the mast cell, or on calcium mobilization or utilization within the mast cell.

This work was supported by a grant from the M.R.C. to Professor G.P. Lewis. The calcium ionophore was kindly supplied by the Lilly Research Centre, Ltd.

**References**

DIAMANT, B. (1975). Energy production in rat mast cells and its role for histamine release. *Int. Archs. Allergy appl. Immun.*, **49**, 155–171.

GARLAND, L.G. & MONGAR, J.L. (1976). Differential histamine release by dextran and the ionophore A23187: the actions of inhibitors. *Int. Archs. Allergy appl. Immun.*, **50**, 27–42.

NORTHOVER, B.J. (1973). Effect of anti-inflammatory drugs on the binding of calcium to cellular membranes in various human and guinea-pig tissues. *Br. J. Pharmac.*, **48**, 496–504.

THOMAS, R.U. & WHITTLE, B.J.R. (1976). Prostaglandins and the release of histamine from rat peritoneal mast cells. *Br. J. Pharmac.*, **57**, 474–475P.

# Effects of prostaglandin synthetase inhibitors and of other drugs on aggregation of plasma-free rabbit platelets by carrageenan and by thrombin

B.B. VARGAFTIG

*Centre de Recherche Merrell International, 16, rue d'Ankara, 67000 Strasbourg, France*

Since hypotension due to carrageenan (Vargaftig, Bhargava, de Vos & Bonta, 1970) and to thrombin (Radegran, 1971) is accompanied by platelet aggregation, we studied the ability of thrombin and carrageenan to aggregate Tyrode-suspended plasma-free rabbit platelets (Vargaftig, Tranier & Chignard, 1974) and to release prostaglandin (PG), rabbit aorta contracting (RCS) and thromboxane-like activities (Born, 1962; Piper & Vane, 1969; Vargaftig & Zirinis, 1973; Bunting, Moncada & Vane, 1976). Thrombin aggregated platelets at 0.005–0.01 u/ml and carrageenan at 0.5–2.5 µg/ml. Carrageenan required 37°C strictly. Inhibition by EGTA and EDTA (2.5–5 mM), by dibutyryl cyclic AMP (0.5–1 mM), by adenosine (1 mM) and by the ADP scavenger apyrase (0.5–2 mg/ml) and failure of ADP itself to induce aggregation, suggested that ADP acted when released intracellularly. Heparin (100 u/ml), tosyl arginine methyl ester (0.5–1 mM) and neuraminidase (10–25 u/ml) inhibited thrombin but not carrageenan-induced aggregation. Neuraminidase lowered the threshold for carrageenan 5–10 fold. Aspirin, indomethacin, salicyclic acid and dinitrophenol (0.1–0.5 mM) suppressed aggregation by threshold amounts of carrageenan or thrombin only. When platelets were washed and resuspended in drug-free plasma or Tyrode, aggregation by carrageenan or thrombin recovered, but PG synthetase inhibition by aspirin and by indomethacin persisted, platelets failing to aggregate and to form PG from arachidonic acid. Dithiothreitol and catalase inhibited carrageenan and thrombin at 5–10 mM and 2.5 mg/ml respectively, concentrations being 10–20 times higher than those needed for inhibition of arachidonic acid (Vargaftig, Tranier & Chignard, 1975). This confirms that intracellular mechanisms are involved with carrageenan and thrombin-induced aggregation, whereas in case of arachidonic acid generation/activity of aggregating substances occur in more accessible sites. Tosylphenylalaninechloromethylketone and bromophenacyl bromide (5–10 µM), inhibitors of chymotrypsin and/or of phospholipase $A_2$ respectively (Schoellman & Shaw, 1963; Volwerk, Pieterson & de Haas, 1974) blocked aggregation by carrageenan and thrombin, but not by ADP or arachidonic acid in plasma. The trypsin inhibitor tosyllysinechloromethylketone was inactive at 0.1 mM. A stable 5-HT-like activity was released from platelets or blood incubated with thrombin (0.5–1.0 u/ml) and carrageenan (25–100 µg/ml). Labile rabbit aorta and coeliac artery contracting activities, inhibited by aspirin and by indomethacin, but not by salicylic acid, were uncovered in platelets from reserpinized rabbits, that aggregated as controls. Cyproheptadine and methysergide (100 µg/ml) failed to inhibit aggregation, ruling out 5-HT as involved directly. Generation of PG by thrombin-stimulated platelets is accompanied by phospholipase release and is suppressed by aspirin (Smith & Willis, 1971), although aggregation by the amounts of thrombin used (5 u/ml) is not inhibitable. Aggregation by carrageenan and thrombin are: (a) PG synthetase-independent and start by separate pathways, (b) proceed by common routes, involving chymotrypsin-like and phospholipase activity (Schoene & Iacono, 1975), as hypothesized for mast cell degranulation (Uvnäs, 1962), and catalase-sensitive substances.

## References

BORN, G.V.R. (1962). Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature (Lond.)*, **193**, 927–929.

BUNTING, S., MONCADA, S. & VANE, J.R. (1976). The effects of prostaglandin endoperoxides and thromboxane $A_2$ on strips of rabbit coeliac artery and certain other smooth muscle preparations. *Br. J. Pharmac.* (in press).

PIPER, P.J. & VANE, J.R. (1969). Relation of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature (Lond.)*, **223**, 29–35.

RÅDEGRAN, K. (1971). Circulatory and respiratory effects of induced platelet aggregation: an experimental study in dogs. *Acta Chir. Scand.*, **Suppl. 420**, 1–24.

SCHOELLMANN, G. & SHAW, E. (1963). Direct evidence for the presence of histidine in the active center of chymotrypsin. *Biochemistry*, **2**, 252–255.

SCHOENE, N.W. & IACONO, J.M. (1975). Stimulation of platelet phospholipase $A_2$ activity by aggregating agents. *Fed. Proc.*, **34**, 790.

SMITH, J.B. & WILLIS, A.L. (1971). Aspirin selectively inhibits prostaglandin production in human platelets. *Nature New Biol.*, **231**, 235–237.

UVNÄS, B. (1962). Mechanism of histamine release in mast cells. *Ann. N.Y. Acad. Sci.*, **103**, 278–284.

VARGAFTIG, B.B., BHARGAVA, N., DE VOS, C.H. & BONTA, I.L. (1970). Interference of carrageenin, non-steroidal anti-inflammatory drugs and protease inhibitors with the kallikrein-kinin system. In *Bradykinin and Related Kinins*, eds. Sicuteri, F. & Rocha e Silva, M., pp. 477–485. New York: Plenum Press.

VARGAFTIG, B.B., TRANIER, Y. & CHIGNARD, M. (1974). Inhibition by sulfhydryl agents of arachidonic acid-induced platelet aggregation and release of potential inflammatory substances. *Prostaglandins*, **8**, 133–156.

VARGAFTIG, B.B., TRANIER, Y. & CHIGNARD, M. (1975). Blockade by metal complexing agents and by catalase of the effects of arachidonic acid on platelets: Relevance to the study of anti-inflammatory mechanisms. *Eur. J. Pharmac.*, **33**, 19–29.

VARGAFTIG, B.B. & ZIRINIS, P. (1973). Platelet aggregation induced by arachidonic acid is accompanied by release of potential inflammatory mediators distinct from $PGE_2$ and $PGF_{2\alpha}$. *Nature New Biol.*, **224**, 114–116.

VOLWERK, J.J., PIETERSON, W.A. & DE HAAS, G.H. (1974). Histidine at the active site of phospholipase $A_2$. *Biochemistry*, **13**, 1446–1454.

## Mitogenic activity of different types of non-immunological inflammatory exudates on macrophages in culture

M. ADOLPHE, C.J. DUNN, J. FONTAGNÉ, J.P. GIROUD, P. LECHAT, D. PAUBLANT & D.A. WILLOUGHBY

*Institut de Pharmacologie, L.A. C.N.R.S. 206, 21, rue de l'Ecole de Médecine, 75006 Paris, France*

*Département de Pharmacologie, E.R.A. C.N.R.S. 629, 27, rue du faubourg Saint-Jacques, 75014 Paris, France*

As described in previous publications from our laboratories, rat peritoneal macrophages in culture could be stimulated to divide after treatment with an acute inflammatory exudate obtained 4 h after intrapleural injection of dextran (Adolphe, Fontagné, Pelletier & Giroud, 1975). Similar results were obtained on mouse macrophages treated with exudate obtained 4 days after implantation of diffusion chambers (Wynne, Spector & Willoughby, 1975).

The present report is an investigation into the effect of different types of acute inflammatory exudates on macrophage DNA synthesis in culture in order to confirm the presence of a 'macrophage mitogenic factor(s)' in other inflammatory reactions. For this purpose the activity of exudates obtained 4 h after intrapleural injection of dextran, λ-carrageenan, κ-carrageenan and calcium pyrophosphate were compared.

All experiments were performed on cultured normal rat peritoneal macrophages. After a 3-day culture period the medium was discarded and the macrophages treated with exudate diluted 50% in culture medium 199 supplemented with 20% calf serum. After a culture period of 3–4 days, macrophages DNA-synthesis was determined by autoradiography and the number of labelled cells counted.

The results show that of the four types of exudate tested all induced increased macrophage DNA-synthesis. However, differences were observed between the activity of these exudates. Firstly exudates induced by λ-and κ-carrageenan, and calcium pyrophosphate caused a greater increase in labelled cells than dextran exudates. This may be indicative of a lower level of 'mitogenic factor' in the dextran exudates, which contains relatively fewer cells and a lower exudate protein concentration compared with carrageenan and calcium pyrophosphate induced exudates. Secondly carrageenan exudates were also observed to stimulate higher binucleate cell formation than in control macrophage cultures.

It is concluded that the observed stimulation of macrophage DNA-synthesis induced by the four types of inflammatory exudate may be due to the release of an inflammatory mitogenic factor during the acute reaction, the production of which may be related to the presence of leucocytes in the exudate.

### References

ADOLPHE, M., FONTAGNÉ, J., PELLETIER, M. & GIROUD, J.P. (1975). Induction of DNA synthesis in rat macrophages *in vitro* by inflammatory exudate. *Nature (Lond.)*, **253**, 637.

WYNNE, K.M., SPECTOR, W.G. & WILLOUGHBY, D.A. (1975). Macrophage proliferation *in vitro* induced by exudates. *Nature (Lond.)*, **253**, 636–637.

---

## Dose-dependent desensitization of lymphocyte beta-receptor function after long-term culture with isoprenaline

M.E. CONOLLY, J.K. GREENACRE & P. SCOFIELD

*Department of Clinical Pharmacology, Royal Postgraduate Medical School*

Szentivanyi (1968) has proposed that asthmatics have an inherent partial blockade of β-adrenoceptor mechanisms. Several investigators (Parker & Smith, 1973; Logsdon, Middleton & Coffey, 1972; Alston, Patel & Kerr, 1974) have reported data from studies of lymphocytes or mixed leukocytes which would appear to support this theory. However, most of the asthmatics studied were receiving bronchodilators acting on β-adrenoceptors and possible effects of this therapy have not been fully considered.

Desensitization to the action of catecholamines by prior exposure to these agents has been shown in fibroblasts (Franklin, Morris & Twose, 1975), adipocytes (Ho & Sutherland, 1971; Smith, Isaakson, Nyberg & Sjöström, 1976), macrophages (Remold-O'Donnel, 1974) and $C_6$ astrocytoma cells (Browning, Brostrom & Groppi, 1976). The present study was designed to examine β-adrenoceptor function in lymphocytes after prolonged exposure to isoprenaline *in vitro.*

Lymphocytes from normal subjects on no medication were cultured in TC 199 for 24 h without and with isoprenaline $1 \times 10^{-8}$ mol/l to $1 \times 10^{-6}$ mol/l. The cells were washed, equilibrated with Hanks balanced salt solution and incubated for 15 min with isoprenaline $1 \times 10^{-4}$ mol/l and theophylline

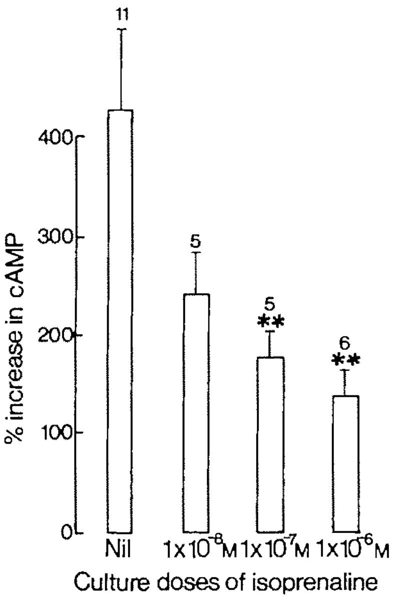


**Figure 1** Mean percentage increase ± s.e. mean in cyclic AMP in response to isoprenaline ($1 \times 10^{-4}$ M) in lymphocytes cyltured without and with isoprenaline. Difference from control: ** significant at the 0.01% level compared to cells with no isoprenaline in culture (paired *t*-test). Figure at top of columns indicates number of experiments.

$1 \times 10^{-2}$ mol/l. Cyclic AMP was assay by the Gilman method (1970) as a measure of $\beta$-adrenoceptor function. Cells cultured with isoprenaline were significantly less responsive to $\beta$-adrenoceptor stimulation in the 15 min incubation and this desensitization increased as cells were cultured with higher concentrations of the drug (see figure). Lymphocytes from three asthmatics were studied similarly and showed no qualitative differences. Cells were also incubated with $PGE_1$. Despite prior culture with isoprenaline, the response to $PGE_1$ at 10.0 µmol/l was not changed, although there was a slight but not significant reduction in the response to $PGE_1$ at 0.5 µmol/l. Phosphodiesterase activity was examined, but was found not to be increased in cells cultured with isoprenaline.

It is concluded that lymphocyte $\beta$-adrenoceptor function, as assayed by cAMP production, can be specifically desensitized by prior exposure to $\beta$-adrenoceptor stimulant.

## References

ALSTON, W.C., PATEL, K.R. & KERR, J.W. (1974). Response of leukocyte adenyl cyclase to isoprenaline and the effect of alpha blocking drugs in extrinsic asthma. *Brit. med. J.*, **i**, 90–93.

BROWNING, E.T., BROSTROM, C.O. & GROPPI, V.E. (1976). Altered adenosine cyclic 3′,5′-monophosphate synthesis and degradation by C-6 astrocytonic cells following prolonged exposure to norepinephrine. *Mol. Pharmacol.*, **12**, 32–40.

FRANKLIN, T.J., MORRIS, W.P. & TWOSE, P.A. (1975). Desensitization of beta-adrenergic receptors in human fibroblasts in tissue culture. *Mol. Pharmacol.*, **11**, 485–491.

GILMAN, A.G. (1970). A protein binding assay for adenosine 3′,5′-cyclic monophosphate. *Proc. natn. Acad. Sci., U.S.A.*, **67**, 305–312.

HO, R.J. & SUTHERLAND, E.W. (1971). Formation and release of a hormone antagonist by rat adipocytes. *J. biol. Chem.*, **246**, 6822–6827.

LOGSDON, P.A., MIDDLETON, E. & COFFEY, R.G. (1972). Stimulation of leukocyte and adenyl cyclase by hydrocortisone and isoproterenol in asthmatic and non-asthmatic subjects. *J. Allergy clin. Immunol.*, **50**, 45–56.

PARKER, C.W. & SMITH, J.W. (1973). Alterations in cyclic adenosine monophosphate metabolism in human bronchial asthma. I. Leukocyte responsiveness to $\beta$adrenergic agents. *J. clin. Invest.*, **52**, 48–59.

REMOLD-O'DONNEL, E. (1974). Stimulation and desensitization of macrophage adenylate cyclase by prostaglandins and catecholamines. *J. biol. Chem.*, **249**, 3615–3621.

SMITH, U., ISAKSSON, O., NYBERG, G. & SJÖSTRÖM, L. (1976). Human adipose tissue in culture. IV. Evidence for the formation of a hormone antagonist by catecholamines. *Europ. J. clin. Invest.*, **6**, 35–42.

SZENTIVANYI, A. (1968). The beta-adrenergic theory of the atopic abnormality in bronchial asthma. *J. Allergy*, **42**, 203–232.

## Non-saturable co-operative binding of tolbutamide to isolated islets of Langerhans

R. ALRIC, F. LIGNON, A.L. LOUBATIÈRES, M. MANTEGHETTI & R. PUECH

*Laboratory of Pharmacology and Pharmacodynamics, Faculty of Medicine of Montpellier, France*

Functionally active islets of Langerhans isolated from rat pancreas with bacterial collagenase and gathered by means of calibrated sieves, bind tritiated tolbutamide (Alric, Manteghetti, Puech, Lignon & Loubatières, 1975). The bound drug was measured as the amount taken up at equilibrium at 37°C in excess of the [$^{14}$C]-sucrose space of the preparation. Both isotopes were measured separately by liquid scintillation following combustion of the samples.

The uptake exhibits some features characteristic of binding to cellular structures rather than diffusion to the intracellular space. In particular, tolbutamide uptake shows a non-saturable co-operative pattern in relation to concentrations. The concentration-binding relationships (binding isotherms) show a steadfast upward concavity up to about $5 \times 10^{-5}$ M, then turn to a straight line.

Such a co-operative pattern indicates that the binding ability of islet cell structures is modified by the binding itself as long as few binding sites are occupied by the drug. This occurs precisely in the range of concentrations which exert a graded stimulating action on the secretion of insulin *in vitro*. At greater concentrations, the binding follows a linear relationship similar to simple physical adsorption on an inert support. Other investigations (Sehlin, 1973) have already shown that this binding finally goes to saturation, but at tolbutamide concentrations far beyond pharmacologically active ones, with a half-maximum at $2 \times 10^{-3}$ M.

Binding of tolbutamide to exocrine pancreas shows much less co-operativity in the same conditions, indicating a great deal of organ specificity. As was expected, unlabelled tolbutamide $5 \times 10^{-5}$ M cancels the co-operativity of binding when added just prior to the labelled drug. A similar effect is produced by chlorpropamide and carbutamide at equiactive concentrations (Alric & Portal, 1969) as well as diazoxide at an equi-antagonistic concentration (Loubatières, Mariani, Alric, Chapal & Portal, 1967). But 'second generation' hypoglycemic sulphonylureas glibenclamide and glipizide at equiactive concentrations, and even glisoxepide, in an equimolar amount, failed to do so. Variations of glucose (1 to $16 \times 10^{-3}$ M) or calcium concentrations did not affect co-operativity of binding.

In spite of the lack of interference of glucose, calcium and second generation sulphonylureas, it remains tempting to consider that the co-operativity of tolbutamide binding to islets could be related to the cell perturbation involved in the stimulating action exerted by this drug on the secretion of insulin by beta cells.

### References

ALRIC, R., MANTEGHETTI, M., PUECH, R., LIGNON, F. & LOUBATIÈRES, A. (1975). Liage coopératif du tolbutamide par les îlots de Langerhans isolés de rat. *C. R. Acad. Sci.*, Série D, **281**, 2029–2032.

ALRIC, R. & PORTAL, A. (1969). Activité pharmacologique des sulfonylurées insulino-sécrétrices étudiée en fonction de leur solubilité aqueuse. *C. R. Soc. Biol.*, **163**, 703–707.

LOUBATIÈRES, A., MARIANI, M.M., ALRIC, R., CHAPAL, J. & PORTAL, A. (1967). Antagonisme entre le diazoxide et le tolbutamide sur l'insulino-sécrétion. Etude 'in vitro' sur le pancréas isolé et perfusé du rat. *C. R. Soc. Biol.*, **161**, 1755–1759.

SEHLIN, J. (1973). Evidence for specific binding of tolbutamide to the plasma membrane of the pancreatic B-cells. *Acta Diabet. Lat.*, **10**, 1052–1060.

---

## Interrelations between MAO activity and carbohydrate metabolism

G. ISMAHAN, H. PARVEZ, S. PARVEZ & A. RAZA-BUKHARI

*Endocrinology Laboratory, University of Paris, Centre of Orsay, Bât 491, 91405 Orsay, France*

The possible interrelations between monoamine oxidase (MAO) activity and metabolism of carbohydrates in central and peripheral regions was studied. Ninety per cent inhibition of MAO activity by administration of deprinil (20 mg/kg) in normally fed male rats resulted in 60% decrease in hepatic glycogen whereas cerebral glycogen stores were completely exhausted but the cardiac glycogen rose by 30% Similar administration of deprinil to rats starved for 24 h produced a three-fold increase in hepatic glycogen but cardiac and cerebral glycogen reserves were slightly affected. Activity of enzyme catechol O-methyl transferase (COMT) in the adrenals of fed rats declined by 47% after selective inhibition of MAO by deprinil but enzyme phenylethanolamine *N*-methyl transferase (PNMT) showed an increase of 20% from non-treated values. Both these enzymes in the adrenals were more severely affected by deprinil in 24 h starved rats since the decrease in COMT and the increase in PNMT were more marked than in the fed animals. Adrenaline stores in the adrenal gland showed an increase but noradrenaline stores declined after deprinil administration to fed rats. The starved rats treated with deprinil demonstrated increased levels of adrenal noradrenaline as well as adrenaline from the value of control rats. Expressing MAO activity per mg of glycogen in each organ and comparing it with MAO per mg of tissue or protein showed direct correlation with variations in glycogen content and MAO activity. The results provide evidence about an important link between the regulatory processes of carbohydrate metabolism and the control of MAO activity in male rats. The use of deprinil also suggests that selective inhibition of MAO activity may have important consequences upon physiological processes of metabolism with respect to glucose and glycogen.

---

## Effects of drugs on suppressed responding

P.B. DEWS

*Laboratory of Psychobiology, Harvard Medical School, Boston, MA, USA*

A type of procedure that has been widely used in studies in behavioural pharmacology has been variously called 'conflict' or 'punishment'. Repetitive responding of the nature of repeated operations of an electrical key by a subject is maintained by a schedule of reinforcement and then, during signalled periods of time, responses are followed by a noxious stimulus according to a program. During the signalled periods, responding is suppressed to a greater or lesser extent, depending on parameter values. This type of procedure has been studied in pigeons, rats and monkeys. There are two main reasons for the popularity of the procedure in pharmacology. First, the results by different investigators from Geller & Seifter (1960) to the present time have been very consistent in showing clearly differential effects of important classes of behaviour-affecting drugs: suppressed responding is increased, i.e. more responding occurs, following effective doses of chlordiazepoxide and barbiturates but not by amphetamine or chlorpromazine which may increase the suppression (Kelleher & Morse, 1968; Dews & DeWeese, 1976). Second, the results lend themselves to plausible descriptions in terms of 'fear' and 'anxiety' that are attenuated by minor tranquillizers.

It has never been demonstrated, however, that the selective effects of the drugs are related to the noxious stimulus (in practice, an electric shock). In the present work responding has been suppressed by a stimulus that is in no way noxious. A rhesus monkey is seated in an isolation chamber with a small lever in comfortable reach in front. When the monkey presses the lever, a white light appears and remains on for 30 s and then goes off. During this period time is being accumulated towards a total interval of between 30 s and 5 min; when that predetermined interval has accumulated, the white light goes out and the next response produces food. The white light therefore merely signals that time is being accumulated toward the moment when food will again be available; nevertheless, the white light suppresses responding. Responding in the presence of the white light is increased by chlordiazepoxide HCl (10–100 µm/kg) and pentobarbital Na (10–30 µM/kg) but not by chlorpromazine HCl (1–10 µM/kg) or methamphetamine HCl (1–10 µM/kg), thus being affected by drugs just like responding suppressed by electric shock. It seems that the selectivity of the drugs depends on the characteristics of the controls of the responding and not on the 'fear' and 'anxiety' engendered by electric shocks and so the commonsense interpretation of 'conflict' and 'punishment' situations is misleading.

### References

GELLER, I. & SEIFTER, J. (1960). The effects of meprobamate, barbiturates, *d*-amphetamine and promazine on experimentally induced conflict in the rat. *Psychopharmacologia*, **1**, 482–492.

KELLEHER, R.T. & MORSE, W.H. (1968). Determinants of the specificity of behavioral effects of drugs. *Ergeb. Physiol.*, **60**, 1–56.

DEWS, P.B. & DeWEESE, J. (1976). Schedules of reinforcement. In *Handbook of Psychopharmacology*, ed. Iversen, L.L., Iversen, S. & Snyder, S.H. New York: Plenum Press. (In press.)

# A comparison of circling behaviour induced in nigro-striatal lesioned rats after peripheral administration of indole derivatives

J.R. BOISSIER, C. DUMONT,
C. OBERLANDER & M. PETERFALVI

*Roussel-Uclaf, B.P. 9, 93230 Romainville, France*

A number of compounds related to tryptamine and three piperidinyl-indole derivatives have been examined for their effects on the circling behaviour in nigro-striatal lesioned rats.

Sprague Dawley rats were electrolytically lesioned in the right striatum (A 8.2, L 3.0, H-0.8) (König & Klippel, 1963) or injected with 6-hydroxydopamine in the right substantia nigra (Ungerstedt, 1971).

The following compounds were tested: tryptamine HCl (10 mg/kg i.p.), 5-methoxytryptamine (5-MT; 5 mg/kg i.p.); 5-methoxy *N*, *N*-dimethyltryptamine (5-MDT; 1 or 2 mg/kg i.p.), (±)-5-hydroxytryptophan (5-HTP; 200 mg/kg i.p.), LSD (0.2 or 1 mg/kg i.p.), methysergide maleate (5 mg/kg s.c.), 3-(4-piperidinyl) 1 H-indole HCl (PI or RU 22424) and the corresponding 5-hydroxy- and 5-methoxy-substituted indole derivatives (5-HPI and 5-MPI or RU 23686), 10 or 20 mg/kg i.p.

In striatal lesioned rats, tryptamine and the 5-substituted derivatives (5-MT, 5-MDT, 5-HTP) were inactive as well as methysergide while LSD caused ipsilateral circling at the high dose used (1 mg/kg). Although PI and 5-HPI had no effect, 5-MPI (10 mg/kg) induced a long lasting contralateral turning behaviour (maximum 3rd h. ipsilateral turns 12 ± 3/contralateral turns 208 ± 26, $n = 36$).

When tested 1 h after scopolamine bromhydrate (10 or 25 mg/kg i.p.) no modification was observed for tryptamine, 5-MT, 5-MDT, and methysergide, while LSD-induced ipsilateral turning was potentiated. In the piperidinyl-indole series scopolamine revealed a strong contralateral rotating behaviour with PI (maximum 3rd h., ipsilateral turns 66 ± 12/contralateral turns 191 ± 22, $n = 22$) similar to the effects of 5-MPI which were prolonged in these conditions.

In nigral lesioned rats the tested compounds were inactive except LSD (0.2 mg/kg) which induced strong contralateral turning and 5-MPI which, at the highest dose only (20 mg/kg) provoked a biphasic response characterized by ipsilateral turning followed by contralateral turning in some animals.

The direction of rotations observed with 5-MPI alone of PI after treatment with a cholinolytic in striatal lesioned rats, and their inefficiency at the same dose in supersensitive 6-hydroxytryptamine lesioned rats are in contradiction to a stimulation of post-synaptic striatal dopamine receptors. Blockade of these receptors is no more likely since PI and 5-MPI do not antagonize apomorphine-induced rotations but on the contrary potentiate them. Moreover there is no clear evidence for a role of tryptaminergic pathways in the mechanism of circling observed with PI and 5-MPI, since 5-HTP, a precursor of 5-HT, and 5-MT or 5-MDT, proposed as 5-HT receptor stimulants do not exhibit any effect in the same experimental conditions.

Just as some experiments have shown that rotations induced by dopamine receptor stimulants were not necessarily the consequence of an impairment in the nigro-striatal tract (Costall & Naylor, 1974; Pycock, Donaldson & Marsden, 1975), the results obtained with the two piperidinyl-indole derivatives clearly suggest that rotations do not solely originate from a classical dopamine receptor stimulating action.

## References

COSTALL, B. & NAYLOR, R.J. (1974). Stereotyped and circling behaviour induced by dopaminergic agonists after lesions of the midbrain raphé nuclei. *Eur. J. Pharmacol.*, **29**, 206–222.

KÖNIG, J.F.R. & KLIPPEL, R.A. (1963). The rat brain: a stereotaxic atlas of the forebrain and lower parts of the brain stem. Baltimore: Williams & Wilkins.

PYCOCK, C.J., DONALDSON, I.MacG. & MARSDEN, C.D. (1975). Circling behaviour produced by unilateral lesions in the region of the locus coeruleus in rats. *Brain Res.*, **97**, 317–329.

UNGERSTEDT, U. (1971). Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. *Acta physiol. scand.*, **82**, Suppl. 367, 69–93.

# A behavioural model of the GABA-facilitating action of benzodiazepines: rotational behaviour after unilateral intranigral injection of chlordiazepoxide

J.L. WADDINGTON
(introduced by T.J. CROW)

*Division of Psychiatry, Clinical Research Centre, Harrow, Middlesex HA1 3UJ*

Recent studies on the mode of action of benzodiazepines have indicated a facilitatory effect on GABA-aminergic transmission (Costa, Guidotti, Mao & Suria, 1975). Rotational behaviour, which has been associated with asymmetric activation of nigrostriatal dopamine (DA) mechanisms, has recently been induced by unilateral manipulation of GABA activity in the substantia nigra zona reticulata (SNR), presumably via a striato-nigral GABA modulation of activity in DA neurones located in the adjacent zona compacta (SNC). Thus blockade of nigral GABA receptors by unilateral intranigral injection of picrotoxin induces contralateral rotation (Tarsy, Pycock, Meldrum & Marsden, 1975), while elevation of nigral GABA level by injection of a GABA transaminase inhibitor produces ipsilateral rotation under the action of amphetamine and apomorphine (Dray, Oakley & Simmonds, 1975). If benzodiazepines exert a facilitation on GABA-minergic transmission then their unilateral injection into SNR should induce ipsilateral rotation.

Male Sprague-Dawley rats (150 ± 20 g) were anaesthetized with ether and unilateral injections of the soluble benzodiazepine chlordiazepoxide hydrochloride (CDP; 1 μg in 1 μl saline) were made stereotaxically into SNR. Control animals received 1 μl saline. Immediately post injection they were placed in automated rotameter bowls and rotation measured continuously for 60 minutes.

Animals receiving CDP injection showed a weak ipsilateral circling/postural deviation on recovery. When pretreated with (+)-amphetamine (5 mg/kg) prior to CDP injection, however, rats showed intense ipsilateral circling that significantly exceeded the mild circling induced by control procedures ($P < 0.01$). Only weak circling was seen after apomorphine pretreatment. With histologically confirmed injections into the region of DA-containing cell bodies (SNC), both CDP and saline induced a mild, indistinguishable, ipsilateral circling. An analysis of variance showed a statistically significant ($P < 0.05$) depth × treatment interaction at 30 minutes. The induction of intense circling in amphetamine pretreated animals by CDP injection was mimicked by injections into SNR of the GABA-analogue baclofen (*β*)-*p*-chlorphenyl)-GABA; 0.1 μg in 1 μl saline; $P < 0.01$ compared with controls). These effects of both CDP and Lioresal injections into SNR were reduced to control levels by i.p. injection of the GABA antagonist picrotoxin (2 mg/kg).

There is some debate on the exact nature of synaptic GABA-DA interactions in SNR (Dray & Straughan, 1975). This CDP effect is, however, mimicked by the GABA-analogue baclofen and both effects are attenuated by the GABA antagonist picrotoxin. These effects are confined to the GABA containing SNR. Such results indicate a facilitatory effect of chlordiazepoxide on GABA transmission and suggest that this rotational model may be useful in the preclinical assessment of benzodiazepines.

J.L.W. is an MRC scholar. Chlordiazepoxide and baclofen were generous gifts of Roche Products Ltd and Ciba Laboratories respectively.

## References

COSTA, E., GUIDOTTI, A., MAO, C.C. & SURIA, A. (1975). New concepts on the mechanism of action of benzodiazepines. *Life Sci.*, **17**, 167–186.

DRAY, A. & STRAUGHAN, D.W. (1976). Synaptic mechanisms in the substantia nigra. *J. Pharm. Pharmac.*, **28**, 400–405.

DRAY, A., OAKLEY, N.R. & SIMMONDS, M.A. (1975). Rotation behaviour following inhibition of GABA metabolism unilaterally in the rat substantia nigra. *J. Pharm. Pharmac.*, **27**, 627–629.

TARSY, D., PYCOCK, C., MELDRUM, B. & MARSDEN, C.D. (1975). Rotational behaviour induced in rats by intranigral picrotoxin. *Brain Res.*, **89**, 160–165.

## The behavioural effects of EOS-induced changes in substantia nigra GABA levels

G. KOOB, MARINA DEL FIACCO & SUSAN D. IVERSEN

*Department of Experimental Psychology, Downing Street, Cambridge*

GABA levels in the substantia nigra (SN) are raised after local injection of ethanolamine-*O*-sulphate (EOS), an inhibitor of GABA: glutamate transaminase (Dray, Oakley & Simmonds, 1975). Dray, Fowler, Oakley, Simmonds & Tanner (1975) have reported lowered striatal dopamine levels on the side of the EOS injection and rotational behaviour consistent with asymmetrical striatal functioning. These effects faded after 5 days. We have studied the changes in spontaneous and amphetamine induced motor behaviour after *bilateral* injection of EOS into the SN (zona reticulata).

Rats are implanted bilaterally with 23 gauge cannulae to within 1 mm of a site in the zona reticulata of the SN. The co-ordinates were A, 3.0; L, 2.0; V, 6.8 from the atlas of Pellegrino and Cushman. For the EOS injection the rat was held firmly and a 30 gauge injection cannula was lowered through the guide to a site 1 mm beyond the guide tip. EOS (200 μg/kg) was injected in 1.5 μl of saline over a 2 min period. Control rats received a 1.5 μl of vehicle solution under identical conditions. Thirty minutes elapsed between the injections on the two sides of the brain. Behaviour was observed immediately following the EOS injection. Twenty-four and 72 h after the injection spontaneous locomotor behaviour was measured for 30 min and stereotyped behaviour was rated. (+)-Amphetamine (1.5 mg/kg) was then administered and these behaviours recorded for 2 hours. The EOS injection was repeated at weekly intervals. The cannulae placements were verified histologically at the end of the experiment.

Immediately following the EOS injection contralateral turning occurred (i.e. away from the injection side). When the injection was given on the second side turning in the opposite direction occurred. Stereotyped behaviours including sniffing and biting were also seen soon after the injection and were still pronounced 24 h later. At this time (+)-amphetamine (1.5 mg/kg) intensified the on-going stereotyped behaviour both in its nature and duration. These effects became more pronounced after each weekly EOS injection.

The results suggest that raising GABA levels in the zona reticulata of the SN results in heightened functional activity of the dopamine-containing nigro-striatal tract. This activity results in spontaneous stereotypy and enhanced motor responses to amphetamine. The nigro-striatal system appears to become progressively sensitized to the EOS treatment.

### References

DRAY, A., OAKLEY, N.R. & SIMMONDS, M.A. (1975). Rotational behaviour following inhibition of GABA metabolism unilaterally in the rat substantia nigra. *J. Pharm. Pharmac.*, **27**, 627–629.

DRAY, A., FOWLER, L.J., OAKLEY, N.R., SIMMONDS, M.A. & TANNER, T. (1975). Comparison of circling behaviour following unilateral inhibition of GABA-transaminase or discrete electrolytic lesioning in the rat substantia nigra. *Br. J. Pharmac.*, **55**, 288P.

---

## Platelet uptake of [$^{14}$C]-5-hydroxytryptamine in 'emotional' and 'non-emotional' rats

J.R. BOISSIER, F. GODEFROY, P. SOUBRIE, M.H. THIEBOT & J. WEIL-FUGAZZA

*Unité de Neuropsychopharmacologie de l'INSERM, 2 rue d'Alésia, 75014 Paris, France*

A method has been developed in our laboratory for selecting rats characterized by a high level of emotionality (Boissier, Simon & Soubrie, 1975). We have undertaken studies in order to look for biochemical differences between 'emotional' (E) and 'non-emotional' (NE) rats. We report here data concerning 5-hydroxytryptamine (5-HT) uptake by blood platelets. Such a study is of interest because the uptake of amines by platelets is thought to be a model for some aspects of aminergic brain function.

The experiment was performed on 14 E rats and 14 NE rats. Two fractions of 1 ml of platelet-rich plasma were prepared for each rat. The uptake of [$^{14}$C]-5-HT (25 nmol, 1 μCi/ml) was measured at suitable time intervals. Statistical analysis (Student's *t* test) of the results (expressed in d.p.m. × $10^3$ per $10^7$ platelets) indicates that 5-HT uptake by platelets is lower in E rats than in NE rats:

| *Time of incubation (min)* | *5* | *15* | *30* |
|---|---|---|---|
| E rats | 1.97 ± 0.13 | 3.60 ± 0.13 | 6.52 ± 0.35 |
| NE rats | 2.00 ± 0.07 | 4.68 ± 0.21 | 7.82 ± 0.40 |
| | ns | $P<0.001$ | $P<0.02$ |

If the mechanism of uptake by platelets is indeed a model for uptake by neurons (Trenchard & Turner, 1975), then our results are indicative of a reduced 5-HT uptake at the neuronal level in E rats. Using the method proposed by Carlsson, Corrodi, Fuxe & Hökfelt (1969), we tried to further evaluate uptake of 5-HT in the brain. According to these authors the rate of 5-HT depletion caused by H75/12 compound (4-methyl-$\alpha$-ethyl-*m*-tyramine) gives an indication of the rate of uptake at the level of the neuronal cell membrane.

The experiment was performed on 12 E rats and 12 NE rats. We observed that, when E rats are treated with H75/12 (25 mg $kg^{-1}$) for 1 h, the brain 5-HT level is decreased 21%. Under the same conditions the brain 5-HT level of NE rats is decreased 43%. The difference between these two percentages is significant ($0.02 < P < 0.05$).

This result is in agreement with the hypothesis that 5-HT uptake at the neuronal level is reduced in E rats. But investigations of uptake by synaptosomal preparations are necessary to provide further support for this hypothesis.

**References**

BOISSIER, J.R., SIMON, P. & SOUBRIE, P. (1975). New approaches to the study of anxiety and anxiolytic drugs in animal. *Proc. VI Intern. Congr. Pharmac.*, **3**, 213–222.

CARLSSON, A., CORRODI, H., FUXE, K. & HÖKFELT, T. (1969). Effect of anti-depressant drugs on the depletion of intraneuronal brain 5-hydroxytryptamine stores caused by 4-methyl-α-ethyl-meta-tyramine. *Eur. J. Pharmacol.*, **5**, 357–360.

TRENCHARD, A. & TURNER, P. (1975). The effects of protriptyline and clomipramine *in vitro* on the uptake of 5-hydroxytryptamine and dopamine in human platelet-rich plasma. *Psychopharmacol.*, **43**, 89–93.

---

## Effects of *p*-chlorophenylalanine and α-methyltryptophan on rat social behaviour

G. CURZON & C.A. MARSDEN

*Department of Neurochemistry, Institute of Neurology, Queen Square, London WC1 3BG*

Previously we have studied the effect on rat motor activity of an inhibitor of 5-hydroxytryptamine (5-HT) synthesis, *p*-chlorophenylalanine (PCPA), and its reversal by the 5-HT precursor tryptophan (Marsden & Curzon, 1976). In the present communication the effects of PCPA on social behaviour are compared with those of $\alpha$-methyltryptophan (AMTP), a drug which induces tryptophan pyrrolase and thus decreases brain 5-HT synthesis by depleting the precursor tryptophan (Sourkes, Missala & Oravec, 1970).

Male Sprague-Dawley rats (110–130 g) were housed 3 to a cage under a 12 h light/dark cycle for ten days. Six cages of rats were then injected with PCPA (200 mg/kg i.p.) and six with (±)-AMTP (150 mg/kg i.p.). Controls were injected with the vehicle medium (0.5% Tween in 0.9% saline). Various components of social behaviour in the home cage were observed for 5 min under red light during the first 90 min of the dark period. Observations were made 24 and 72 h after injection. Locomotor activity was measured simultaneously using an Animex activity meter. The amount of food eaten/24 h was also measured. Either 24 or 72 h after drug treatment the rats were killed and brain 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) determined (Curzon & Green, 1970).

Both drugs reduced food intake during the first 24 h after injection but it was normal by 72 hours. PCPA markedly reduced 5-HT synthesis at 24 and 72 h with a significant decrease of 5-HT (24 h, –42%; 72 h, –53%) and 5-HIAA (24 h, –51%; 72 h, –67%). This was associated at 24 h (but not at 72 h) with increased locomotor activity ($P < 0.05$) and increased interactions between rats involving sniffing and biting ($P < 0.01$), fighting ($P < 0.01$) and mounting ($P < 0.01$). Self-grooming was decreased ($P < 0.05$). AMTP also decreased 5-HT turnover as brain 5-HIAA was reduced (24 h, –41%; 72 h, –43%) but 5-HT concentrations appeared to increase presumably due to the formation of $\alpha$-methyl-5-HT (Roberge, Missala & Sourkes, 1972). In contrast to PCPA, AMTP had no significant effect on any of the above components of social behaviour or net locomotor activity. Self-grooming was increased at 24 h ($P < 0.01$) and at 72 h ($P < 0.05$) and burrowing was increased at 24 h ($P < 0.01$). $\alpha$-Methyl-5-HT is formed by the action of tryptophan hydroxylase (Gal & Christiansen, 1975). To investigate whether $\alpha$-methyl-5-HT was involved in the behavioural effects of AMTP tryptophan hydroxylase was inhibited by injecting PCPA (100 mg/kg) on three consecutive days to 24 rats and

AMTP (150 mg/kg) was then given to 12 of these rats 24 h after the last dose of PCPA. Twenty-four hours later there were no significant differences in social behaviour between PCPA and PCPA + AMTP treated rats.

The results suggest that when rats are given AMTP the formation of $\alpha$-methyl-5-HT opposes the behavioural effects of decreased 5-HT synthesis.

**References**

CURZON, G. & GREEN, A.R. (1970). Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of rat brain. *Br. J. Pharmac.*, **39**, 653–655.

GAL, E.M. & CHRISTIANSEN, P.A. (1975). $\alpha$-Methyltryptophan: Effects on cerebral mono-oxygenases *in vitro* and *in vivo*. *J. Neurochem.*, **24**, 89–95.

MARSDEN, C.A. & CURZON, G. (1976). Studies on the behavioural effects of tryptophan and *p*-chlorophenylalanine. *Neuropharmacology*, **15**, 165–171.

ROBERGE, A.G., MISSALA, K. & SOURKES, T.L. (1972). $\alpha$-Methyltryptophan: Effects on synthesis and degradation of serotonin in the brain. *Neuropharmacology*, **11**, 197–209.

SOURKES, T.L., MISSALA, K. & ORAVEC, M. (1970). Decrease of cerebral serotonin and 5-hydroxyindolylacetic acid caused by (—)-alpha-methyltryptophan. *J. Neurochem.*, **17**, 111–115.

---

## Reversal of the action of $\gamma$-aminobutyric acid (GABA) antagonists by barbiturates

N.G. BOWERY

*Department of Pharmacology, St Thomas' Hospital Medical School, London SE1 7EH*

Certain hypnotic barbiturates have been reported to activate receptors for the central inhibitory neurotransmitter $\gamma$-aminobutyric acid (GABA) in the isolated frog spinal cord (Nicoll, 1975a, b). Also they prolong post-synaptic inhibition in the feline hippocampus which may be mediated by GABA (Nicholl, Eccles, Oshima & Rubia, 1975).

Sympathetic ganglion cells possess GABA-receptors which are analogous to those in the mammalian brain; activation of these receptors produces an easily measured depolarization (Bowery & Brown, 1974). This provided a model for testing the effect of barbiturates on GABA receptors.

Ganglion cell depolarization in the isolated desheathed superior cervical ganglion of the rat was recorded using extracellular $Ag^+/AgCl$ electrodes. The ganglia were superfused at 1 ml/min with Krebs solution at 25°C containing hyoscine (2.6 µM) (Brown & Marsh, 1975). GABA (1–300 µM) or carbachol (15–100 µM) were applied alternately for 1 min periods at 10–15 min intervals. Both produced a dose-dependent depolarization.

A depolarizing response to sodium pentobarbitone occurred only at high concentrations (>80 µM) and this was of low amplitude. A more striking effect, which could be observed at lower concentrations, was the reversal of the action of GABA-antagonists as shown in Figure 1a. When responses to GABA were reduced by bicuculline methochloride the simultaneous addition of sodium pentobarbitone restored responses to GABA. Pentobarbitone applied alone depressed responses to GABA and carbachol.

Pentobarbitone reversed the effects of other GABA antagonists, picrotoxin, tetramethylene-disulphotetramine (Bowery, Brown & Collins, 1975) and isopropyl bicyclo phosphate (Bowery, Collins & Hill, 1976). In contrast it did not reverse the antagonistic effect of hexamethonium against carbachol (Figure 1b). This selectivity for GABA was also apparent with strychnine: this depressed responses to GABA and carbachol equally (Bowery & Brown, 1974) but only the antagonism to GABA was reversed by pentobarbitone.

Thiopentone and amylobarbitone were as active as pentobarbitone whereas hexobarbitone and butobarbitone were less potent. Barbitone and phenobarbitone were inactive at concentrations up to 400 µM.

**References**

BOWERY, N.G. & BROWN, D.A. (1974). Depolarizing actions of $\gamma$-aminobutyric acid and related compounds on rat superior cervical ganglia *in vitro*. *Br. J. Pharmac.*, **50**, 205–218.

BOWERY, N.G., BROWN, D.A. & COLLINS, J.F. (1975). Tetramethylene-disulphotetramine: an inhibitor of $\gamma$-

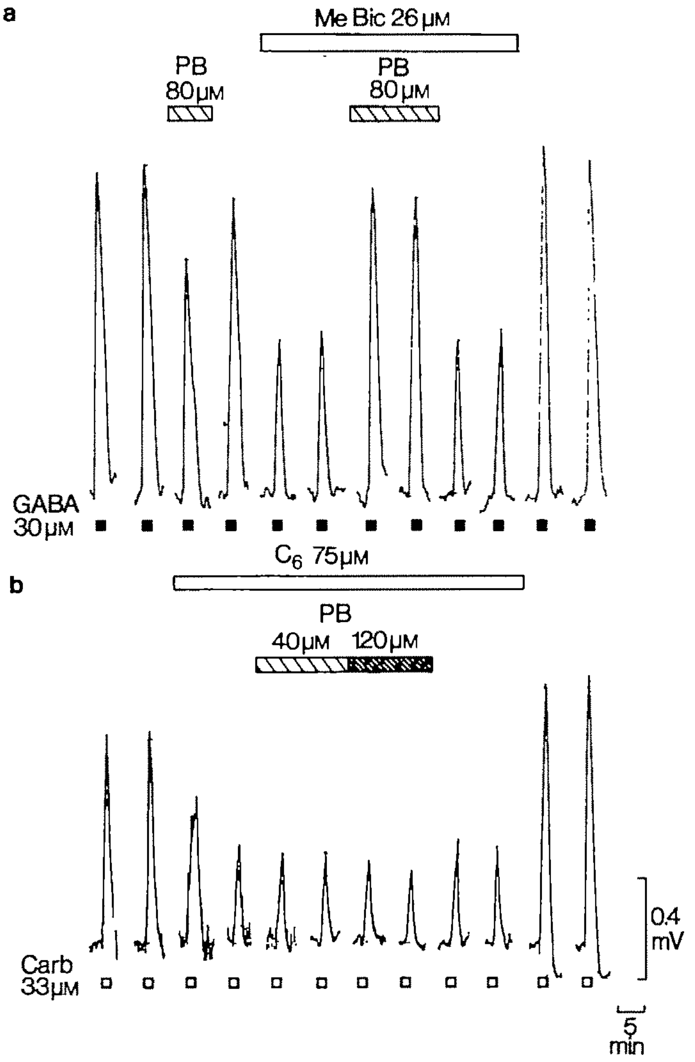


**Figure 1** Depolarizing responses to GABA (30 µM, a) and carbachol (33 µM, b) in two isolated superior cervical ganglia of the rat. (a) Reversal by sodium pentobarbitone (PB. 80 µM) of the depression in responses to GABA produced by bicuculline methochloride (MeBic. 26 µM). (b) Lack of effect of sodium pentobarbitone (40 and 120 µM) on the antagonism of responses to carbachol produced by hexamethonium ($C_6$ 75 µM). Gaps indicate periods >20 minutes.

aminobutyric acid induced depolarization of the isolated superior cervical ganglion of the rat. *Br. J. Pharmac.*, **53**, 422–424.

BOWERY, N.G., COLLINS, J.F. & HILL, R.G. (1976). Bicyclic phosphorus esters that are potent convulsants and GABA antagonists. *Nature (Lond.)*, **261**, 601–603.

BROWN, D.A. & MARSH, S. (1975). A very simple method for recording ganglion depolarization. *J. Physiol. (Lond.)*, **246**, 24–26P.

NICOLL, R.A. (1975a). Presynaptic action of barbiturates in the frog spinal cord. *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1460–1463.

NICOLL, R.A. (1975b). Pentobarbital: action on frog motoneurons. *Brain Res.*, **96**, 119–123.

NICOLL, R.A., ECCLES, J.C., OSHIMA, T. & RUBIA, F. (1975). Prolongation of hippocampal inhibitory postynaptic potentials by barbiturates. *Nature (Lond.)*, **258**, 625–627.

## Evidence for naloxone and opiates as GABA antagonists

E. BREUKER, R. DINGLEDINE &
L.L. IVERSEN

*MRC Neurochemical Pharmacology Unit, Department of Pharmacology, Cambridge*

In several species the behavioural effects of opiates change from inhibition to excitation and finally convulsions, as the dose is increased. We report here several lines of evidence that the convulsant properties of drugs with chemical structures related to morphine, and in particular naloxone, may be the result of GABA receptor blockade. For each experiment male T.O. Strain mice 25–30 g were randomly divided into five groups of eight, and used only once. Bicuculline, strychnine HCl and naloxone HCl were administered intraperitoneally, and the presence of convulsions (end point: bilateral clonic or tonic extensions of hindlimbs) was noted over the following 30 minutes. The data were analysed by the method of Litchfield & Wilcoxon (1949). Naloxone caused convulsions in mice, with an ED$_{50}$ of 190 mg/kg (as the base). Pretreatment of mice with a subconvulsant dose of naloxone (90 mg/kg i.p. 5 min before challenge) significantly ($P<0.05$) reduced the ED$_{50}$ for bicuculline convulsions (potency ratio$=$2.2), while not affecting the dose-response for strychnine (potency ratio$=$1.1). On the other hand, diazepam pretreatment (5 mg/kg i.p. 30 min before challenge) significantly increased the ED$_{50}$ of both bicuculline and naloxone (in both cases potency ratio$=$0.5) while not affecting strychnine-induced convulsions (potency ratio$=$1.2).

In other experiments, bicuculline, naloxone, morphine, levorphanol and dextrorphan were found to displace [$^3$H]-GABA from GABA receptor sites in homogenates of human cerebellum, using the GABA binding assay of Enna & Snyder (1975). Each drug was tested with 4–6 concentrations, each in duplicate. The IC$_{50}$s (in μM) for the above drugs were 8, 308, 400, 250 and 300, respectively.

Finally, naloxone was tested for its ability to block the inhibition evoked by iontophoretically applied GABA on single neurones in the rat nucleus accumbens and olfactory tubercle. Multibarrelled pipettes (tips 4–6 μ) were filled with 4 M NaCl (recording and current balancing barrels), 1 M GABA pH 4.8 and 50 mM naloxone in 100 mM NaCl, pH 5.0. When tested in this way, naloxone applied by microiontophoresis completely and reversibly antagonized GABA-evoked inhibition in 6 of 10 neurones.

It is postulated that the behavioural excitation seen after administering large amounts of some opiates or opiate antagonists may reflect functional blockade of GABA-inhibitory systems.

### References

ENNA, S.J. & SNYDER, S.H. (1975). Properties of γ-aminobutyric acid (GABA) receptor binding in rat brain synaptic membrane fractions. *Brain Res.*, **100**, 81–97.

LITCHFIELD, J.T. & WILCOXON, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmac. exp. Ther.*, **96**, 99–113.

---

## Actions of enkephalin and morphine on spinal cord and brain stem neurones

J. DAVIES & A. DRAY

*Department of Pharmacology, The School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX*

Enkephalin occurs in brain tissue as two pentapeptides, methionine (ME) and leucine enkephalin (LE) (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975) and has been suggested to be the endogenous ligand for the opiate receptor in the CNS (Kosterlitz & Hughes, 1975). While this compound mimics the actions of morphine at peripheral sites (Hughes, 1975; Hughes *et al.*, 1975) and possesses analgesic activity when administered intracerebroventricularly (Belluzzi, Grant, Garsky, Sarantakis, Wise & Stein, 1976) its effects on individual central neurones are unknown.

Conventional electrophysiological and micro-electrophoretic techniques have been used to investigate the actions of synthetic methionine and leucine enkephalin on single neurones in the spinal cord of the pentobarbitone anaesthetized cat and in the pons-medulla of the urethane anaesthetized rat. The same multi-barrelled glass micropipettes were used in experiments on each species.

In the spinal cord, morphine consistently excited Renshaw cells but not other non-cholinoceptive interneurones (Duggan, Davies & Hall, 1976). This property was shared by enkephalin, ME being more consistent in this respect than LE. Both morphine and enkephalin-induced excitation was reversibly reduced by the narcotic antagonist naloxone. Neither morphine nor enkephalin depressed the firing rate of any of the spinal neurones studied, however, enkephalin selectively reduced acetylcholine-induced but not morphine-induced excitation of Renshaw cells. By contrast, morphine enhanced the actions of acetylcholine and amino acid excitants on these neurones and also antagonized the depressant action of glycine on spinal neurones whereas enkephalin did not modify this action of glycine.

The effects of morphine and enkephalin on brain stem neurones were qualitatively similar to those observed on spinal cord neurones. Hence, excitation was the predominant effect of enkephalin and morphine on brain stem neurones. These neurones could also be excited by acetylcholine. Occasionally, enkephalin and morphine caused a reduction in cell firing rate. As in the spinal cord ME was a more effective excitant than LE. Morphine enhanced the excitatory effect of acetylcholine and reduced glycine-induced depression whereas enkephalin reduced the response to acetylcholine and did not affect glycine depression.

These observations provide direct evidence that enkephalin acts on central opiate receptors and are consistent with the hypothesis that enkephalin is the endogenous ligand for the stereospecific opiate receptor (Kosterlitz & Hughes, 1975). However, it is not clear whether the additional properties of enkephalin, not shared by morphine, are of physiological importance.

This work was supported by an MRC programme grant to Professor D.W. Straughan. We are grateful to Dr B.A. Morgan (Reckitt and Colman Ltd) for gifts of enkephalin and Endo Laboratories Inc. for naloxone hydrochloride.

**References**

BELLUZZI, J.D., GRANT, N., GARSKY, V., SARANTAKIS, D., WISE, C.D. & STEIN, L. (1976). Analgesia induced *in vivo* by central administration of enkephalin in rat. *Nature (Lond.)*, **260**, 625–626.

DUGGAN, A.W., DAVIES, J. & HALL, J.G. (1976). Effects of opiate agonists and antagonists on central neurones of the cat. *J. Pharmac. exp. Ther.*, **196**, 107–120.

HUGHES, J. (1975). Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. *Brain Res.*, **88**, 295–308.

HUGHES, J., SMITH, T.W., KOSTERLITZ, H.W., FOTHERGILL, L.A., MORGAN, B.A. & MORRIS, H.R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature (Lond.)*, **258**, 577–579.

KOSTERLITZ, H.W. & HUGHES, J. (1975). Some thoughts on the significance of enkephalin, the endogenous ligand. *Life Sci.*, **17**, 91–96.

---

●

## The effects of morphine and met-enkephalin on nociceptive neurones in the rat thalamus

R.G. HILL & C.M. PEPPER

*Department of Pharmacology, University of Bristol, Bristol BS8 1TD*

Morphine applied iontophoretically has been found to both excite and depress the activity of single central neurones (Bradley & Dray, 1974; Satoh, Zieglgänsberger & Herz, 1975; Duggan, Davies & Hall, 1976). In contrast, we have found that iontophoretically applied met-enkephalin (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975) is predominantly depressant (Hill, Pepper & Mitchell, 1976). We have therefore investigated the actions of morphine and met-enkephalin on the same population of neurones and, to increase the relevance of the study to analgesic mechanisms, we have confined our study to nociceptive neurones located in the rat thalamus.

Adult male rats were anaesthetized with 1% halothane in oxygen. Single unit recordings were made from the central barrel of a 5-barrelled micropipette stereotaxically placed in the thalamus. Recording and current balancing barrels were filled with 4 M NaCl. Other barrels contained combinations of L-glutamate (0.5 M, pH 8.5), GABA (0.5 M, pH 3.0) morphine hydrochloride (50 mM, no adjustment of pH) and synthetic met-enkephalin (8 mM, no adjustment of pH) for iontophoretic application. Techniques for extracellular recording of action potentials and iontophoresis of drugs were conventional. Nociceptive neurones were identified by their consistent increases in firing rate following noxious stimulation of the tail (Hellon & Mitchell, 1975). Stimuli used were immersion of the tail in hot (50–55°C) water for 30–45s or a strong pinch with a thermometer clamp. Twenty-two nociceptive neurones were identified in 16 rats.

Intravenous morphine (hydrochloride; 0.6 to 5.0 mg/kg) prevented or greatly reduced the increase in firing rate produced by noxious stimulation. This effect was typically rapid in onset (<2 min) and of long duration (up to 70 min). Intravenous naloxone (hydrochloride; 0.15 to 0.6 mg/kg) reversed this action of morphine. Similar results have been obtained by others in studies of nociceptive neurones in the dorsal horn of the cat spinal cord (Le Bars, Menetrey, Conseiller & Besson, 1975).

The change in the firing rate of the thalamic neurones in our study may have been secondary to a change in the presynaptic volley exciting them, since the discharge in ventrolateral tract axons is much reduced by intravenous morphine (Jurna & Grossman, 1976). However, direct iontophoretic application of either morphine (10–40 nA) or met-enkephalin (0–100 nA) to nociceptive neurones was found to depress both spontaneous and glutamate evoked activity and to prevent the excitant effects of noxious stimulation. No evidence of excitation of these neurones by morphine was seen.

Thus, on this restricted population of neurones at least, the actions of morphine and enkephalin are similar. This investigation would therefore lend support to the suggestion (Kosterlitz & Hughes, 1975) that enkephalin may function in the brain as an endogenous morphine-like factor controlling the appreciation of noxious stimuli.

This work was supported by the MRC and C.M.P. is an MRC scholar. We would like to thank Dr B. Morgan of Reckitt & Colman Ltd., for the gift of synthetic met-enkephalin.

**References**

BRADLEY, P.B. & DRAY, A. (1974). Morphine and neurotransmitter substances: micro-iontophoretic study in the rat brain stem. *Br. J. Pharmac.*, **50**, 47–55.

DUGGAN, A.W., DAVIES, J. & HALL, J.G. (1976). Effects of opiate agonists and antagonists on central neurones of the cat. *J. Pharmac. exp. Ther.*, **196**, 107–120.

HELLON, R.F. & MITCHELL, D. (1975). Characteristics of neurones in the ventrobasal thalamus of the rat which respond to noxious stimulation of the tail. *J. Physiol. (Lond.)*, **250**, 29–30P.

HILL, R.G., PEPPER, C.M. & MITCHELL, J.F. (1976). The depression of nociceptive and other neurones in the rat brain by iontophoretically applied met-enkephalin. *Nature (Lond.)* (in press).

HUGHES, J., SMITH, T.W., KOSTERLITZ, H.W., FOTHERGILL, L.A., MORGAN, B.A. & MORRIS, H.R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature (Lond.)*, **258**, 577–579.

JURNA, I. & GROSSMAN, W. (1976). The effect of morphine on the activity evoked in ventrolateral tract axons of the cat spinal cord. *Exp. Brain Res.*, **24**, 473–484.

KOSTERLITZ, H.W. & HUGHES, J. (1975). Some thoughts on the significance of enkephalin, the endogenous ligand. *Life Sci.*, **17**, 91–96.

LE BARS, D., MENETREY, D., CONSEILLER, C. & BESSON, J.M. (1975). Depressive effects of morphine upon lamina V cells activities in the dorsal horn of the spinal cat. *Brain Res.*, **98**, 261–277.

SATOH, M., ZIEGLGÄNSBERGER, W. & HERZ, A. (1975). Interaction between morphine and putative excitatory neurotransmitters in cortical neurones in naive and tolerant rats. *Life Sci.*, **17**, 75–80.

---

# Interactions of peptides derived from the C-fragment of β-lipotropin with brain opiate receptors

N.J.M. BIRDSALL, A.F. BRADBURY,
A.S.V. BURGEN, E.C. HULME,
D.G. SMYTH & C.R. SNELL

*National Institute for Medical Research, Mill Hill, London, NW7 1AA*

The pentapeptide sequence of methionine–enkephalin (met–E, Tyr-Gly-Gly-Phe-Met) is also the N-terminal sequence of the C-Fragment of β-lipotropin, a 31 residue peptide which is found in considerable quantity in the pituitary (Bradbury, Smyth & Snell, 1975). This peptide has a high affinity for brain opiate receptors, as measured by competition with [$^3$H]-naloxone and [$^3$H]-dihydromorphine for binding to a washed membrane preparation. The potency of C-Fragment is equally great against the two tritiated opiates, a characteristic of morphinoid antagonists, but not of agonists (Pert & Snyder, 1974) or methionine–enkephalin, which display greater potency against dihydromorphine than against naloxone (Bradbury, Smyth, Snell, Birdsall & Hulme, 1976).

The removal of residues 30 and 31 (Gly-Gln) from C-Fragment has little effect on the binding properties, but further removal of residues 28 and 29 (Lys-Lys) reduces the affinity by a factor of 20 (Bradbury *et al.*, 1976). This suggests that interaction of C-terminal residues, including lysines 28 and 29, with the receptor may make an important contribution to the binding of C-Fragment, both augmenting the affinity and modifying the binding properties of the N-terminal pentapeptide sequence. Direct evidence for the existence of a binding site for the C-terminal comes from the observation that the tridecapeptide, composed of residues 19–31, inhibits [$^3$H]-naloxone and [$^3$H]-dihydromorphine binding, with an IC$_{50}$ of $3 \times 10^{-6}$M, despite the absence of the met–E sequence.

The binding site for the N-terminal region of C-Fragment has been probed by investigating the binding properties of a series of analogues of met–E. N-methylation of met–E, or conversion of the carboxyl terminal to an amide group produces a 2–4 fold increase in the affinity without affecting the morphine-like binding properties of met–E. However, N-carbamylation, benzylation of the tyrosine hydroxyl group, or substitution of the 2-glycine residue by l-proline, or l-alanine all drastically reduce binding affinity. Substitution of sarcosine in the 2-

position reduces the potency against [$^3$H]-naloxone thirty-fold, but produces a much larger effect on the potency against [$^3$H]-dihydromorphine.

Although the binding properties of C-Fragment resemble those of the alkaloid antagonists, it is a potent central analgesic (Feldberg & Smyth, 1976), and acts as a full agonist on the guinea pig ileum (Hughes & Kosterlitz, personal communication). The presence of two binding sites for C-Fragment may account for this unexpected combination of properties.

The authors wish to thank Pramila Mehta and Derek Massey for skilled technical assistance.

**References**

BRADBURY, A.F., SMYTH, D.G. & SNELL, C.R. (1975). In *Peptides: chemistry, structure and biology*. Proc. IV American Peptide Symposium, ed. Meienhofer, J. pp. 61–65. Ann Arbor: Ann Arbor Science Inc.

BRADBURY, A.F., SMYTH, D.G., SNELL, C.R., BIRDSALL, N.J.M. & HULME, E.C. (1976). C-Fragment of lipotropin has a high affinity for brain opiate receptors. *Nature (Lond.)*, **260**, 793–795.

FELDBERG, W.S. & SMYTH, D.G. (1976). The C-Fragment of lipotropin—a potent analgesic. *J. Physiol. (Lond.)* (in press).

PERT, C.B. & SNYDER, S.H. (1974). Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Mol. Pharmacol.*, **10**, 868–879.

# DEMONSTRATIONS

## Prostaglandin $E_2$ released from the kidneys of genetic hypertensive rats contributes to the vasoconstrictor supersensitivity to angiotensin II *in vitro*

J.M. ARMSTRONG & N. LATTIMER

*Wellcome Research Laboratories, Beckenham, Kent*

Prostaglandin $E_2$ ($PGE_2$) causes vasodilation in the kidneys of dogs or cats and when synthesized and released in response to angiotensin II (AII, attenuates the magnitude of the AII vasoconstriction (Aiken & Vane, 1973; Needleman, Johnson, Jakschik, Douglas & Marshall, 1974). However, in rat kidneys, $PGE_2$ or its precursor arachidonic acid produces vasoconstriction (Malik & McGiff, 1975). An exaggeration of this vasoconstriction due to a relative lack of 15-hydroxyprostaglandin dehydrogenase could be the primary inherited abnormality responsible for hypertension in inbred New Zealand hypertensive (GH) rats (Armstrong, Blackwell, Flower, McGiff, Mullane & Vane, 1976). We have now investigated the possible interactions of AII ($Val^5$ hypertension II amide, Ciba or $Val^3$ $Ile^5$ Cambrian Chemicals) and angiotensin III (AIII) (Des-aspartate AII Ciba; AIII Cambrian Chemicals) with $PGE_2$ or arachidonic acid in the renal vascular bed of normotensive and GH rats.

Kidneys of male GH rats (indirect systolic blood pressure 160–190 mmHg) weighing 220–260 g and of 17–19 weeks of age were used and compared with those of normotensives (Otago Wistar or Charles River Wistar, 110–130 mmHg) of similar sex and body weight. Both kidneys of each animal were perfused at 20 ml/min *in vitro* and the PGE-like activity in the effluent bioassayed as previously described (Armstrong *et al.*, 1976).

Intra-arterial infusion of arachidonic acid (100–1000 ng/ml) or injections of AII (1.25 ng–80 ng) increased perfusion pressure indicating vasoconstriction. In addition, AII and arachidonic acid released PGE-like activity as shown by contractions of the assay tissues, especially the chick rectum which is insensitive to AII. The vasoconstrictor response to AII or arachidonic acid in preparations from GH rats was approximately 2 times greater than in kidneys from normotensives. Smaller amounts of AII were needed in kidneys from GH rats for the release of PGE-like activity (2.5–5.0 ng) than in preparations from normotensive animals (5–10 ng). AIII also released PGE-like activity, but the kidneys of either group were less sensitive to its vasoconstrictor effects, needing 4 times the dose to give a comparable effect to AII. Indomethacin infusions (1–2 μg/ml) reduced the magnitude of the vasoconstriction to either AII or AIII, prevented the appearance of the PGE-like activity and abolished vasoconstriction induced by arachidonic acid.

These findings indicate that the released PGE-like substance which accompanies the renal vasoconstriction to AII or AIII augments the response. Both the augmentation and the vasoconstriction are greater in kidneys from hypertensive rats. The failure of exogenous $PGE_2$ (0.1–1.0 ng) in the presence of indomethacin, to potentiate the vasoconstriction to AII is in contrast to findings with noradrenaline (Armstrong *et al.*, 1976) and suggests that AII stimulates vascular sites different from those stimulated by noradrenaline, requiring endogenously synthesized PGs for augmentation. As the kidneys of GH rats were still more sensitive to AII than were the normotensive rats when prostaglandin synthesis had been inhibited by indomethacin, other factors, e.g. structural changes in vessel walls, may also contribute.

### References

AIKEN, J.W. & VANE, J.R. (1973). Intrarenal prostaglandin release attenuates the renal vasoconstrictor activity of angiotensin. *J. Pharmac. exp. Ther.*, **184**, 678–687.

ARMSTRONG, J.M., BLACKWELL, G.J., FLOWER, R.J., McGIFF, J.C., MULLANE, K.M. & VANE, J.R. (1976). Genetic hypertension in rats is accompanied by a defect in renal prostaglandin catabolism. *Nature (Lond.)*, **260**, 582–586.

MALIK, K.V. & McGIFF, J.C. (1975). Modulation by prostaglandins of adrenergic transmission in the isolated perfused rabbit and rat kidney. *Circ. Res.*, **36**, 599–609.

NEEDLEMAN, P., JOHNSON, E.M., JAKSCHIK, B., DOUGLAS, J.R. & MARSHALL, G.R. (1974). Renal hormonal interactions and their pharmacological modification. Renin-angiotensin, catecholamines and prostaglandins. In *Recent Advances in Renal Physiology and Pharmacology*, ed. Wesson, L.G. & Fanelli, G.M. pp. 197–214. Lancaster: M.T.P.

# A device for controlling perfusion pressure

D.C. CARDINAL & T.W.K. HILL
(introduced by J.M.G. WALKER)

*Pharmacology Laboratory, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS*

A control unit has been developed for the perfusion of vascular beds. It will maintain a chosen constant perfusion pressure or cause it to follow variations in systemic arterial pressure. The control unit (Figure 1) is used in conjunction with a polygraph (Beckman R411) for recording systemic arterial and perfusion pressures and a roller pump (Watson-Marlow, MHRE 200).

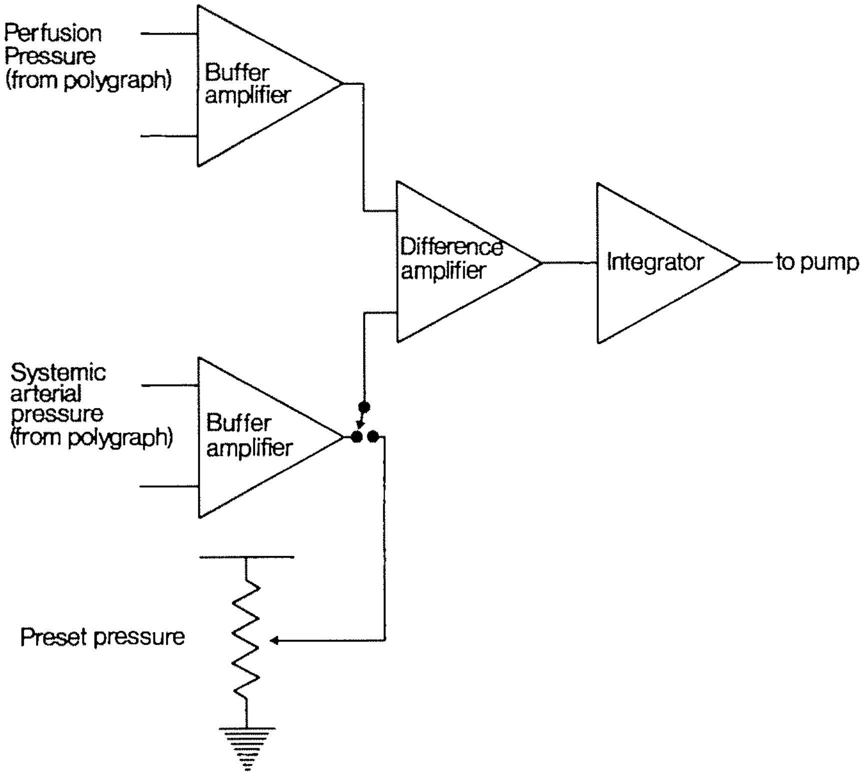


**Figure 1** Control unit.

Voltages transduced from the recorded pressure signals are taken from the polygraph pre-amplifiers and fed into buffer amplifiers to prevent any interaction between the control unit and the polygraph. Comparison between the voltages derived from the perfusion pressure and either the systemic arterial pressure or the manually preset, constant pressure, enables the difference to be detected and amplified. This difference is fed into an integrating amplifier, the output of which provides a voltage for controlling the pump via its own control circuit.

The instrument is an improvement on previous electronic designs (Lowe, 1966; Critchley, Ungar & Whelpdale, 1974) in that it is compact and easy to operate and allows the pump to operate over its full speed range. It is also an improvement on the Starling pneumatic resistance method (Ono, Kokubun & Hashimoto, 1974) as it does not require cannulation of an extra vessel for the shunting of blood.

### References

CRITCHLEY, J.A., UNGAR, A. & WHELPDALE, P.H. (1974). A feed-back amplifier for constant pressure perfusion with a roller pump. *Br. J. Pharmac.*, **52**, 155P.

LOWE, R.D. (1966). A constant pressure variable flow blood perfusion system. *J. Physiol. (Lond.)*, **186**, 45P.

ONO, H., KOKUBUN, H. & HASHIMOTO, K. (1974). Abolition by calcium antagonists of the autoregulation of renal blood flow. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **285**, 201–207.

# A simple and inexpensive method for the intracerebral administration of drug solutions to the conscious rat

P. DAWES & P.H. REDFERN

*School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY*

Many different cannula systems have been designed to allow the direct application of drugs to discrete brain areas of conscious animals (Myers, 1971; Harvey & Stevens, 1975). The major technical problem lies in securing the device to the skull and consequently many of the cannula systems are complex and hence expensive. The system detailed here is both inexpensive and simple to construct.

Cannula guides consist of 23 swg stainless steel tubing cut to the required length and electrochemically cleaned of burr. The length of each guide is obviously dictated by the topographical location of the brain area under investigation but certain minimum requirements are apparent. Thus, 4 mm is sufficient to allow positioning in the stereotaxic cannula-guide carrier and a further 4 mm for cementation. This portion carries a small ball of epoxy-resin (2–3 mm diameter) which acts as guide anchorage and prevents movement of the tube in the vertical plane. The remaining part of the cannula guide which lies below the cranium is of length such that the tip rests 2–3 mm above the area under investigation.

Stilettes, which remain within the cannula-guides at all times other than when injecting, are also simply constructed. Stilette shafts of 30 swg, non-ferrous wire are coated with a water repelling agent (dimethyldichlorosilane) and bent, at one end, over a 3–4 mm length of polythene tubing (I/D 0.86 mm) and secured in place with a 3 mm ball of epoxy resin. This facilitates removal of the stilette from the guide, and the cylinder of polythene, which fits closely over the guide tube provides anchorage and prevents access of foreign material into the underlying tissue. The tip of the shaft lies flush with that of the cannula guide.

The cannula guides are stereotaxically positioned and then secured to the surface of the cranium with polymethyl methacrylate. Sufficient cement is used such that both the guide anchorage and the whole of the incised area are covered. The cement is dried by a stream of warm air for 3–4 min before unclamping the guide from the stereotaxic apparatus. Stilettes are then fitted and the animals housed individually for 5 days before experimentation.

Drug solutions are injected through a 30 swg cannula at a rate of 0.47 μl/min and in a volume of 0.5 μl. Injection placements have been histologically verified following the infusion of 0.5 μl of 1% methylene blue and 4 days storage of tissues in 8% formyl saline at 4°C. Placements were found to be consistently reproducible.

## References

HARVEY, A.H. & STEVENS, R.J. (1975). Construction and implantation of a cannula system for repeated injections into localized regions of the rat brain. *J. Pharm. Pharmac.*, 27, 43–45.

MYERS, R.D. (1971). Methods for Chemical Stimulation of the Brain. In *Methods in Psychobiology*, ed. Myers, R.D. pp. 247–280. London & New York: Academic Press.

---

# Central and peripheral inhibition of the milk-ejection reflex: studies with μ-adrenoceptor antagonists

G. CLARKE & D.W. LINCOLN

*Department of Anatomy, The Medical School, University of Bristol*

Most species of mammal fail to milk eject when they are anaesthetized and suckled by their young. This could be the result of high circulating levels of adrenaline, either blocking the release of oxytocin from the neurohypophysis or preventing the action of the hormone on the mammary gland (Cross, 1955). Rats are an exception and will milk eject when anaesthetized (Lincoln, Hill & Wakerley, 1973), though an appreciable number, too many for some forms of experimental study, still fail to respond to the suckling stimulus. The rat mammary gland is sensitive to the actions of adrenaline, but the effects can be eliminated by β-adrenoceptor antagonists (Bisset, Clark & Lewis, 1967). Thus we have questioned whether central or peripheral adrenoceptor stimulation might explain the failure, or partial failure, of some anaesthetized rats to milk eject when suckled.

Rats, at day 7–10 of lactation and separated from their young for 16 h, were anaesthetized with urethane (1.2 g/kg, i.p.), and two teat ducts were cannulated for the measurement of intramammary pressure. Three hours later, and while the animals were still deeply anaesthetized, 10 pups were applied to the uncannulated nipples. Each milk ejection in the next 3 h was recorded; each ejection was associated with an abrupt rise in intramammary pressure and a behavioural response from the young.

Of 166 rats studied, 90 began to milk eject within 60 min of the young being applied to the nipples and thereafter gave milk ejections at regular intervals. Propranolol (1 mg/kg, i.v. or i.p.) was given to 49 of those which did not milk eject within 1 h, and 42 (86%) subsequently milk ejected. Of 27 rats that were left untreated only 3 (11%) began to milk eject. By contrast, practolol (1 mg/kg, i.v.) promoted milk ejection in only 1 of 10 animals—8 of the 9 failures milk ejected after being given propranolol. Intravenous infusions of adrenaline or isoprenaline (0.1–1.0 μg/min) abolished the milk-ejection reflex but simultaneously reduced the mammary response to exogenous oxytocin; both these actions were antagonized by propranolol. It is unlikely, however, that the failure of some rats to milk eject when anaesthetized and suckled is due to circulating adrenaline depressing the gland response. Such animals display a normal sensitivity to exogenous oxytocin, i.e. compared with animals which do milk eject. Thus, it would appear that the failure of rats to milk eject is the result of a central dysfunction, possible involving an adrenergic mechanism though not necessarily related to circulating amines.

This work was supported by a grant from the Medical Research Council.

### References

BISSET, G.W., CLARK, B.J. & LEWIS, G.P. (1967). The mechanism of the inhibitory action of adrenaline on the mammary gland. *Br. J. Pharmac.*, **31**, 550–559.

CROSS, B.A. (1955). Neurohormonal mechanisms in emotional inhibition of milk ejection. *J. Endocr.*, **12**, 15–28.

LINCOLN, D.W., HILL, A. & WAKERLEY, J.B. (1973). The milk-ejection reflex of the rat: an intermittent function not abolished by surgical levels of anaesthesia. *J. Endocr.*, **57**, 459–476.

---

## Effects of ethanol and chlordiazepoxide on social interaction in rats

SANDRA E. FILE, J. HYDE & M. POOL
(introduced by M.J. NEAL)

*Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX*

Although it is widely held that small doses of ethanol increase social behaviour in man, there is little experimental evidence to support this. In general ethanol has been found to decrease social behaviour in animals (e.g. Krsiak & Borgesova, 1973), but this may well be due to the large doses used. Ethanol (0.4 g/kg) was found to increase exploration in rats (File, unpublished observations) and it was thought that this dose might also increase social behaviour. In order to investigate whether any such increases could be related to anxiety reduction, social interaction between pairs of male rats was studied in conditions in which the level of fear was manipulated. This was done by altering the intensity of illumination and the rats' familiarity with the situation.

In the low fear condition ethanol (0.4 g/kg) did not change the duration of active social contact, but in conditions of moderate fear this dose increased active contact. In the high fear condition active contact was not significantly increased. A higher dose (1.2 g/kg) reduced active contact in all the test conditions, but this may have been secondary to motor impairments.

The effect of the anxiolytic drug, chlordiazepoxide, on social interaction was also studied in the same conditions. Chlordiazepoxide produced a dose related (2.5–7.5 mg/kg) decrease in active social contact and an increase in passive contact.

Both ethanol (0.4 g/kg) and chlordiazepoxide (5 mg/kg) increase exploration in rats, measured by head-dipping in a hole-board, and this effect might be due to anxiety reduction. However, the two drugs differ in their effects on social interaction. The effects of ethanol are consistent with anxiety reduction but the effect of an acute injection of chlordiazepoxide appears to resemble that of a sedative. However, rats pretreated with chlordiazepoxide (5 mg/kg) for 5 days and then tested for social interaction showed significantly increased active contact in conditions of moderate and high fear.

We are grateful to the Science Research Council for supporting this research with a grant to M.J.N. J.H. is a Science Research Council Scholar.

### Reference

KRSIAK, M. & BORGESOVA, M. (1973). Effect of alcohol on behaviour of pairs of rats. *Psychopharmacologia*, **32**, 201–209.

# Active transport of adenine and adenosine by blood platelets and cultured endothelial cells

D.P. de BONO, J.L. GORDON,
D.E. MacINTYRE & J.D. PEARSON

*Departments of Surgery and Pathology, University of Cambridge*

Blood platelets accumulate adenine and adenosine by active transport (Sixma, Holmsen & Trieschnigg, 1973; Sixma, Trieschnigg & Holmsen, 1973), and adenosine uptake is inhibited by dipyridamole (Rozenberg, Ledwidge, Wilcken & McKeon, 1971). We have tested a range of drugs as potential inhibitors of these transport processes, extended the studies to endothelial cells in culture, and developed a cytotoxicity assay based on adenine uptake.

Adenine and adenosine uptake by platelets was measured in 0.1 ml samples of human citrated platelet-rich plasma at 37°C, incubating for 3 min with 0.1 $\mu$M (8-$^3$H) adenine or for 12 min with 0.5 $\mu$M (U-$^{14}$C) adenosine.

Adenine transport was unaffected by a range of thio reagents with different actions: *N*-ethyl maleimide (NEM), dithiothreitol, iodoacetate, 5,5′-dithiobis (2-nitrobenzoic acid), and 2-amino (4-isothioureylmethylene)-thiazol dihydrochloride (Ag 307) did not significantly inhibit uptake at concentrations up to 3 mM. Adenosine transport was inhibited only by NEM ($IC_{50} = 0.2$ mM). Dipyridamole and its derivatives 2,6-bis(diethanolamino)4-piperidino-pyrimido-(5,4d)-pyrimidine (RA 233), 2-(2 amino-ethylamino)4-morpholinothieno-(3, 2d)-pyrimidine dihydrochloride (VK 744) and 2-piperazino-thieno-(3,2d)-pyrimidine dihydrochloride (VK 774) had little effect on adenine uptake at concentrations up to 0.3 mM, but dipyridamole and RA 233 (though not VK 744 or VK 774) inhibited adenosine transport by 80% at 50 $\mu$M. Prostaglandins $D_2$, $E_1$, $E_2$ and $F_{2\alpha}$ did not affect either transport process at concentrations up to 10 $\mu$M.

Porcine endothelial cells in culture (de Bono, 1974) were plated in 6 mm diameter wells ($10^4$ cells/well; 0.2 ml medium). Uptake of (8-$^3$H) adenine was linear for at least 30 min at 0.1 $\mu$M and 5 $\mu$M. The transport process was saturable, with values for $K_m$ and $V_{max}$ of approximately 0.8 $\mu$M and 2.5 nmol per $10^6$ cells/h respectively. The uptake of adenine was unaffected by the presence of 20% v/v autologous heat-inactivated serum. Preliminary experiments have established that these cells also actively transport adenosine, this process being inhibited by NEM (0.1 mM), and abolished by dipyridamole (50 $\mu$M) or in the presence of 20% heat-inactivated serum.

Adenine uptake was reduced in endothelial cells damaged by antibody and complement, or by allergized lymphocytes, and this reduction correlated well both in time of onset and degree of effect with morphologically assessed cytotoxicity.

We have found that fibroblasts and smooth muscle cells in culture also transport adenine, and it seems likely that the active uptake of purine bases is an essential attribute of mammalian cells. The development of the terminal adenine uptake assay described above may therefore prove valuable as a means of assessing cytotoxicity in a wide range of cell types.

Ag 307 was kindly donated by Dr H. Laborit, CEPBEPE, Paris; RA 233, VK 744 and VK 774 by Dr J.W. Bell, Boehringer, Ingelheim; and prostaglandins by Dr J.E. Pike, Upjohn, Kalamazoo. These studies were supported by grants from the Medical Research Council and the Arthritis and Rheumatism Council.

**References**

DE BONO, D.P. (1974). Effects of cytotoxic sera on endothelium *in vitro*. *Nature (Lond.)*, **252**, 83–84.

ROZENBERG, M.C., LEDWIDGE, C.M., WILCKEN, D.E.L. & McKEON, M. (1971). The inhibition of adenosine phosphorylation in platelets by dipyridamole. *J. Lab. clin. Med.*, **77**, 88–96.

SIXMA, J.J., HOLMSEN, H. & TRIESCHNIGG, A.C.M. (1973). Adenine nucleotide metabolism of blood platelets. VIII. Transport of adenine into human platelets. *Biochim. biophys. Acta*, **298**, 460–468.

SIXMA, J.J., TRIESCHNIGG, A.M.C. & HOLMSEN, H. (1973). Adenosine uptake in intact human platelets. IV. *Int. Congress Thromb. Haem.*, Abstract 357.

# Adrenaline on isotonic contractions of mammalian skeletal muscle

D.M. LEWIS & SANDRA N. WEBB

*Department of Physiology, University of Bristol*

The effect of adrenaline on the tension and time course of isometric twitches of mammalian skeletal muscle depends on the speed of contraction of the muscle. In fast-twitch muscle adrenaline increases both the maximal twitch tension and the times to peak tension and half relaxation; in slow-twitch muscle the opposite occurs. In neither muscle is the rate of tension development significantly affected (Bowman & Nott, 1969).

Although these changes are due to direct action on muscle fibres little is known of the mechanisms responsible. The increase in time to peak tension in fast-twitch muscle without significant change in the rate of development of tension suggests an action on the $Ca^{2+}$ release/re-uptake mechanisms rather than changed activity of the contractile proteins. The changes in slow-twitch muscle suggest a similar site of action. However, adrenaline is known to affect the properties of the contractile proteins in cardiac muscle as measured by changes in the force/velocity characteristics during isotonic contractions.

We have tested this point directly by measuring isotonic shortening velocities at various loads in fast-twitch flexor digitorum longus (F.D.L.) muscle and slow-twitch soleus muscle of the cat during continuous i.v. infusion of adrenaline.

Experiments were performed on seven cats anaesthetized with nembutal. Adrenaline was infused into the jugular vein and pressure in the common carotid artery was recorded in all experiments. Isometric contractions were recorded from either flexor digitorum longus or soleus muscles stimulated indirectly once every 10 seconds. After loaded isotonic shortening was measured by a rotary transducer (Birdsall, Buller & Kean, 1971).

Maximal effects of adrenaline on isometric twitches were produced at infusion rates of 5 $\mu g\ kg^{-1}\ min^{-1}$ in flexor digitorum longus and by 1 $\mu g\ kg^{-1}\ min^{-1}$ in soleus. These effects on twitches plus those on isometric and isotonic tetani are given in Table 1.

Adrenaline had no systematic effect on the shortening velocity of either fast-twitch or slow-twitch skeletal muscle of the cat.

These findings indicate an effect on $Ca^{2+}$ release/re-uptake mechanisms. One aspect of this, which could be easily investigated, would operate through a change in the muscle action potential.

**Table 1** Percentage change in contraction characteristics

| | *Flexor digitorum longus* | *Soleus* |
|---|---|---|
| Isometric twitch | | |
| Peak tension | 19.6 (1.88)** | −5.5 (1.41)* |
| Time to peak | 16.6 (1.89)** | −12.6 (0.96)** |
| Time to half relaxation | 22.5 (1.87)** | −20.3 (1.72)** |
| Rate of rise of tension | 8.7 (3.6)* | 4.8 (2.88) |
| Isometric tetanus | | |
| Maximum tension ($P_0$) | 2.9 (2.49) | −2.0 (2.12) |
| Maximum rate of rise of tension | −1.3 (5.3) | −1.2 (1.64) |
| Isotonic tetanus | | |
| Maximum velocity | 2.1 (3.83) | 3.5 (6.8) |
| $a/P_0$ | −13.1 (7.01) | −3.6 (11.6) |

Figures in brackets are s.e. mean.
Changes significant at the 0.05 level are indicated as * and at the 0.001 level as **.

**References**

BIRDSALL, D.L., BULLER, A.J. & KEAN, C.J.C. (1971). A displacement transducer for measuring muscle shortening. *J. Physiol. (Lond.)*, **213**, 11P.

BOWMAN, W.C. & NOTT, M.W. (1969). Actions of sympathomimetic amines and their antagonists on skeletal muscle. *Pharmac. Rev.*, **21**, 27–72.

# Antagonism of responses to anorectics by selective receptor blockers

Z.L. KRUK, L.A. SMITH &
M.R. ZARRINDAST

*Department of Pharmacology and Therapeutics, The London Hospital Medical College, Turner Street, London E1 2AD*

Anorectic drugs are believed to exert their effects by interaction with specific neurochemical mechanisms in the central nervous system. The catecholamines and 5-hydroxytryptamine have become associated with mediating the anorectic response. *In vitro* evidence shows that anorectics have varying abilities to block the uptake and cause the release of dopamine, noradrenaline and 5-HT (Kruk & Zarrindast, 1976). On the basis of this work, it is suggested that it may be possible to separate anorectic drugs into two groups, those acting through a dopaminergic mechanism, and those acting through a 5-HT mechanism. Such *in vitro* evidence for an involvement of brain monoamines in anorectic responses is indirect however and cannot be used to predict anorectic activity with certainty, as it is only possible to measure anorexia *in vivo*. We have therefore examined the ability of selective receptor blocking compounds to antagonize the anorexia observed in rats following treatment with anorectics, in the hope that this will show which receptors are involved in mediating responses to different anorectics. Pimozide (0.25 mg/kg) a dopamine receptor antagonist significantly increased food intake (saline/saline 69.7 ± 5.3 g 6 rats$^{-1}$ h$^{-1}$; pimozide/saline 90.5 ± 4.0 g 6 rats$^{-1}$ h$^{-1}$, $P < 0.5$). Methergoline (0.5 mg/kg) a 5-HT receptor antagonist did not significantly affect food intake (saline/saline 60.8 ± 5.8 g 6 rats$^{-1}$ h$^{-1}$; methergoline/saline 66.0 ± 3.5 g 6 rats$^{-1}$ h$^{-1}$). Phenoxybenzamine (5 mg/kg) significantly decreased food intake (saline/saline 60.7 ± 4.5 g 6 rats$^{-1}$ h$^{-1}$; phenoxybenzamine/saline 46.7 ± 4.2 g 6 rats$^{-1}$ h$^{-1}$ $P < 0.05$) as also did l-propranolol (10 mg/kg) (saline/saline 71.4 ± 4.7 g 6 rats$^{-1}$ h$^{-1}$; l-propranolol/saline 57.5 ± 4.9 g 6 rats$^{-1}$ h$^{-1}$ $P < 0.05$).

The anorectic effects of (+)-amphetamine, mazindol, phenmetrazine and diethyl propion were antagonized by pretreatment with pimozide, and phenmetrazine excepted, not by the other agents. The anorectic effects of fenfluramine and norfenfluramine were antagonized by pretreatment with methergoline, but not by other agents.

The results are broadly in agreement with biochemical data on uptake and release of monoamines, suggesting that anorectics exert their actions through either dopaminergic or 5-HT mechanisms.

**Table 1** The effect of selective receptor blockers on the anorectic responses in groups of six trained rats. Anorectics were administered in the dose ranges indicated 30 min before presentation of food, following pretreatment with receptor blockers at the doses and times indicated. Results are $ED_{50}$ values (with 95% confidence limits) (mg/kg i.p.) defined as that dose needed to decrease food intake to 50% of control in the first hour following presentation of food. $ED_{50}$ values were calculated by the method of Litchfield & Wilcoxon (1949)

| | | *$ED_{50}$ (with 95% confidence limits) mg/kg i.p. following pretreatment with:* | | | | |
|---|---|---|---|---|---|---|
| *Compound* | *Dose range mg/kg* | *Saline (5 ml/kg) 1 or 2 h* | *Pimozide (0.25 mg/kg) 2 h* | *Methergoline (0.5 mg/kg) 2 h* | *Phenoxybenzamine (5 mg/kg) 1 h* | *(–)-Propranolol (10 mg/kg) 1 h* |
| (+)-Amphetamine $SO_4$ | 0.25–8 | 0.9(1.4–0.5) | 1.8(2.9–1.1)* | 1.0(1.4–0.7) | 1.0(1.8–0.6) | 1.05(1.9–0.6) |
| Mazindol | 0.5–16 | 2.4(5.3–1.1) | 6.9(10.4–4.6)* | 2.0(4.6–0.9) | 2.4(4.1–1.4) | 1.0(1.5–0.7)* |
| Diethyl propion HCl | 0.5–48 | 7.7(10.8–5.5) | 16.0(25.3–10.1)* | 3.2(4.8–2.1) | 6.0(9.0–2.8) | 7.0(12.3–4.0) |
| Phenmetrazine HCl | 1.0–64 | 13.5(19.1–9.5) | 36.0(51.7–25.0)*† | 11.5(19.2–6.9)* | 23.0(32.5–16.3)* | 17.5(25.5–12.0) |
| Fenfluramine HCl | 0.5–32 | 6.2(8.4–4.6) | 6.6(11.4–3.8) | 20.0(35.4–11.3)* | 8.0(14.2–4.5) | 3.6(7.9–1.6) |
| Norfenfluramine HCl | 0.25–16 | 2.6(3.4–2.0) | 2.7(4.0–1.8) | 4.4(6.6–2.9)* | 2.4(3.7–1.6) | 1.2(2.4–0.6)* |

* $P < 0.05$.
† Pimozide 0.5 mg/kg i.p. (2 h).

**References**

KRUK, Z.L. & ZARRINDAST, M.R. (1976). The effect of anorectic drugs on uptake and release of brain monoamines. *Br. J. Pharmac.* (in press).

LITCHFIELD, J.T. & WILCOXON, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmac. exp. Ther.*, **96**, 99–113.

---

## Interaction of clonidine with dopamine-dependent behaviours in rodents

P.G. JENNER & C.J. PYCOCK

*University Department of Neurology, Institute of Psychiatry and King's College Hospital Medical School, Denmark Hill, London SE5 8AF*

The central pharmacology of clonidine (2-(2,6-dichlorophenylamino)-2-imidazoline) is complex. It is generally believed to stimulate central α-adrenoceptors (Andén, Corrodi, Fuxe, Hökfelt, Hökfelt, Rydin & Svensson, 1970), although more recent work suggests that it may also modulate central tryptaminergic mechanisms (Maj, Mogilnicka & Palider, 1975).

Since both noradrenergic and 5-hydroxytryptaminergic systems have been shown to modify dopaminergic mechanisms, we have investigated the ability of clonidine to alter dopamine-dependent behaviours in rodents. Clonidine (range 0.06–2 mg/kg) potentiated circling behaviour induced by both apomorphine (0.25 mg/kg, s.c.) and amphetamine (3 mg/kg, i.p.) in mice with unilateral destruction of the nigro-striatal dopaminergic pathway. Similarly, this dose range of clonidine enhanced apomorphine (2 mg/kg)-induced reversal of reserpine akinesia in mice. The drug also potentiated apomorphine-induced hyperactivity resulting from bilateral injections (10 μg) into the nucleus accumbens of rats. Clonidine (100 μg) into one striatum of rats produced no postural asymmetry or circling behaviour, nor was this pretreatment evoked into active turning activity in the presence of systematically administered apomorphine (0.5 mg/kg, s.c.). Clonidine (0.5 mg/kg, i.p.) was without effect on apomorphine (0.1–5 mg/kg, s.c.)-induced stereotypy in rats, but did enhance the catalepsy induced by haloperidol (0.1–2 mg/kg, i.p.) in rats.

This study suggests that clonidine significantly modifies all dopamine-dependent behaviours exhibiting a motor component, *viz*, circling behaviour and locomotor activity. It failed to apparently influence stereotypy or to directly affect striatal dopaminergic mechanisms. Although clonidine potentiated the cataleptic effect of a neuroleptic, its action is likely to be one of non-specific sedation rather than one of a true synergistic monoaminergic mechanism.

Whatever the mechanism of action of clonidine, be it through a noradrenergic, tryptaminergic or any other neuronal system, it appears that such actions do not influence all forms of dopamine mediated behaviour.

CJP is a Fellow of the Parkinson's Disease Society.

**References**

ANDÉN, N.-E., CORRODI, H., FUXE, K., HÖKFELT, B., HÖKFELT, T., RYDIN, C. & SVENSSON, T. (1970). Evidence for a central noradrenaline receptor stimulation by clonidine. *Life Sci.*, **9**, 513–523.

MAJ, J., MOGILNICKA, E. & PALIDER, W. (1975). Serotoninergic mechanism of clonidine and apomorphine interaction. *Pol. J. Pharmacol.*, **27**, 27–35.

# Indomethacin and acid secretion in the isolated perfused canine stomach

MARIE-ANNE PILOT
(introduced by A. D'MELLO)

*Department of Experimental Surgery, The London Hospital Medical College, Ashfield Street, London E1 2AD*

The isolated canine stomach will not secrete acid with a disc oxygenator in the arterial inflow. Green & Auden (1972) and Kowalewski & Sharf (1971), therefore, used a donor dog to oxygenate the blood perfusing the stomach. A completely isolated perfused preparation of the stomach would have many advantages since a donor dog introduces additional variable factors. The following experiments were devised to investigate why acid secretion is inhibited in the isolated perfused stomach.

Greyhounds of both sexes were anaesthetized with intravenous pentobarbitone (20 mg/kg) after premedication with fentanyl (0.24 mg) and droperidol (12 mg), and heparinized. Blood was drained from the femoral artery into the perfusion circuit. The coeliac artery was clamped and the arterial supply to the stomach was perfused with a pulsatile pump via the splenic artery. A cannula in the portal vein shunted venous blood from the stomach to the femoral vein, thus by-passing the liver. A continuous intra-arterial infusion of histamine acid phosphate (20 µg/min) to the stomach was used to stimulate acid secretion.

The stomach secreted acid continuously only when the oxygenator was by-passed. When it was included in the circuit, acid secretion was abolished and the platelet count fell from about 30,000 to 10,000/mm$^3$. It is possible that platelet aggregates were formed in the oxygenator and caused mechanical and chemical damage (Bø & Hognestad, 1972) to the gastric mucosa. In order to remove the aggregates before isolating and perfusing the stomach, blood was circulated for 1 h from the femoral artery through the perfusion circuit including the oxygenator and back into the femoral vein. When the stomach was isolated and perfused after this preliminary procedure, a large acid output (up to 36 mEq/h) was observed in response to histamine.

However, if the venous outflow which normally returned to the heart and lungs was diverted to the oxygenator to establish complete vascular isolation, there was an exponential fall in secretion. This inhibition could have been caused by the release of prostaglandins and their accumulation in the absence of the lungs. These substances, which are metabolized in the lungs (Ferreira & Vane, 1967), can inhibit gastric secretion (Robert, Phillips & Nezamis, 1968). Therefore indomethacin, which inhibits prostaglandin synthesis, was injected intravenously (10 mg/kg) into the dog 30 min before isolation of the stomach. After vascular isolation, the stomach was removed and placed in a chamber where acid secretion continued for two hours. This suggests that indomethacin prevents prostaglandins from accumulating in the perfusate and inhibiting gastric secretion.

Indomethacin, besides permitting sustained acid secretion, also caused extensive bleeding from the gastric mucosa into the lumen of the stomach. This preparation might be useful not only for studies of gastric secretion, but also to study the mechanisms involved in drug-induced gastric bleeding.

## References

BØ, G. & HOGNESTAD, J. (1972). Effects on the pulmonary circulation of suddenly induced intravascular aggregation of blood platelets. *Acta physiol. scand.*, **85**, 523–531.

FERREIRA, S.H. & VANE, J.R. (1967). Prostaglandins. Their disappearance from and release into the circulation. *Nature (Lond.)*, **216**, 868–873.

GREEN, W.E.R. & AUDEN, R.R. (1972). Compartmental blood flow and acid secretion in the intact, and the isolated perfused canine stomach. *Arch. Fr. Mal. App. Digestif*, **61**, 296c.

KOWALEWSKI, K. & SHARF, R. (1971). Secretion of hydrochloric acid by the *ex vivo* isolated canine stomach. *Scand. J. Physiol.*, **6**, 675–681.

ROBERT, A., PHILLIPS, J.P. & NEZAMIS, J.E. (1968). Inhibition by prostaglandin $E_1$ of gastric secretion in the dog. *Gastroenterology*, **54**, 1263.

# The biological significance of prostaglandin-like substances released from immunologically challenged guinea-pig lungs

J.R. BOOT, W. DAWSON & D.J. OSBORNE

*Lilly Research Centre Limited, Erl Wood Manor, Windlesham, Surrey GU20 6PH*

Nine prostaglandin (PG)-like substances have been identified in the perfusate from challenged sensitized guinea-pig lungs perfused *in vitro*. They account for 94% of the total PG-like material released (1.3–1.8 μg/pair of lungs). These figures were derived from 3 groups of 200 guinea pigs and were compared with a non-sensitized group of animals challenged and extracted similarly. No PG-like material was detected in the control perfusate.

These compounds have been tested on a variety of smooth muscle preparations derived from gut and respiratory tree, on pulmonary perfusion pressure *in vitro*, systemic blood pressure on anaphylactic mediator release and on chemotaxis.

Three of the substances, $PGF_{2\alpha}$ (0.6%), 15-oxo $PGF_{2\alpha}$ (6%) and 6-oxo $PGF_{1\alpha}$ (11%) have spasmogenic activity, particularly on respiratory smooth muscle, and each increased pulmonary vascular resistance. 6-oxo $PGF_{1\alpha}$ had $\frac{1}{2}$–$\frac{1}{3}$ the activity of $PGF_{2\alpha}$ in both systems whilst 15-oxo $PGF_{2\alpha}$ had $\frac{1}{10}$–$\frac{1}{5}$ the activity. 6-oxo $PGF_{1\alpha}$ is a novel PG identified in these laboratories (Dawson, Boot, Cockerill, Mallen & Osborne, 1976) and its biological activity is of considerable interest. $PGE_2$ (1%) and 15-oxo $PGE_2$ (trace) were the only substances detected which were capable of relaxing bronchial smooth muscle, the latter having the activity of $PGE_2$.

Thromboxane $B_2$ ($TxB_2$), released from challenged chopped lung preparations (Hamberg & Samuelsson, 1974) was also released in this system (30%) and was minimally active on smooth muscle preparations at high (10 μg/ml) concentrations. A metabolite of $TxB_2$, 15-oxo 13,14-dihydro $TxB_2$ was also identified (42%) but was devoid of spasmogenic activity. This metabolic conversion of $TxB_2$ was confirmed using guinea-pig lung homogenates and a high speed supernatant preparation derived from this tissue, both of which converted purified $TxB_2$ to its metabolite. There was no indication of either the 15-oxo or 13,14-dihydro compounds analogous to the metabolites of the parent PGs.

The 15-oxo 13,14-dihydro metabolites of $PGE_2$ (trace) and $PGF_{2\alpha}$ (3%) were isolated but were without activity in any of the systems studied.

$PGF_{2\alpha}$ (0.5 μg/ml) and $TxB_2$ (1–5 μg/ml), increased the synthesis and release of slow reacting substance in anaphylaxis (SRS-A) from challenged guinea-pig chopped lung preparations whilst $PGE_2$ (0.5 μg/ml) reduced SRS-A release. Sufficient $TxB_2$ is released on challenge to achieve this concentration in the lung.

There are at least two components of this immunological reaction in which PG-like substances are involved: direct bronchoconstrictor activity of the PGs and the modification of mediator release. Of the nine compounds, only $TxB_2$ has been shown to be chemotactic (Boot, Dawson & Kitchen, 1976) and could perhaps develop the inflammation commonly associated with allergic responses in the lung. The interaction with the adenyl and guanyl cyclase systems has not been studied but this could be a possible third component of PG involvement (Lichstenstein, Gillespie, Bourne & Henney, 1972).

## References

BOOT, J.R., DAWSON, W. & KITCHEN, E.A. (1976). The chemotactic activity of Thromboxane $B_2$, a possible role in inflammation. *J. Physiol. (Lond.)*, **257**, 47–48P.

DAWSON, W., BOOT, J.R., COCKERILL, A.F., MALLEN, D.N.B. & OSBORNE, D.J. (1976). The release of novel prostaglandins and thromboxanes after immunological challenge of guinea-pig lung. *Nature (Lond.)*, (in press).

HAMBERG, M. & SAMUELSSON, B. (1974). Prostaglandin endoperoxides. VII. Novel transformations of arachidonic acid in the guinea-pig lung. *Biochem. Biophys. Res. Commun.*, **61**, 942–949.

LICHSTENSTEIN, L.M., GILLESPIE, E., BOURNE, H.R. & HENNEY, C.S. (1972). The effects of a series of prostaglandins on *in vitro* models of the allergic response and cellular immunity. *Prostaglandins*, **2**, 519–528.

## A sponge implantation test in the rat as a model for screening anti-inflammatory activity

J.P. BOLAM, A.W. FORD-HUTCHINSON, M.J.H. SMITH & J.R. WALKER

*Department of Biochemical Pharmacology, King's College Hospital Medical School, Denmark Hill, London SE5 8RX*

Despite the large number of tests currently available to screen potential anti-inflammatory and antirheumatic drugs (Swingle, 1974) there are very few based on leucocyte migration into inflammatory exudates. The implanted sponge technique (Saxena, 1960) seems worthy of more general use since it is a simple procedure, allows a variety of measurements to be performed on exudates at various time intervals after implantation and is suitable for the assessment of inhibitory effects on granuloma formation (Ford-Hutchinson, Smith, Elliott, Bolam, Walker, Lobo, Badcock, Colledge & Billimoria, 1975; Wiener, Wiener, Urivetzky, Shafer, Isenberg, Janov & Meilman, 1975; Clarke, Vernon-Roberts & Currey, 1975).

The method may be of particular value in the assessment of substances which interfere with either the formation or action of leucotactic factors derived from complement since rats depleted of complement components by purified cobra venom factor showed a marked inhibition of cellular migration into the sponge exudate (Wiener, Lendvai, Rogers, Urivetsky & Meilman, 1973).

Some applications of the sponge method to the study of the sites of action of conventional antirheumatic drugs and the relationship between the involvement of blood platelets, leucotaxis and prostaglandins in the inflammatory exudates will be presented.

This work was supported by the King's College Hospital and Medical School Research Committee, the Wates Foundation and the National Research Development Corporation.

**References**

CLARKE, A.K., VERNON-ROBERTS, B. & CURREY, H.L.F. (1975). Assessment of anti-inflammatory drugs in the rat using subcutaneous implants of polyurethane foam impregnated with dead tubercle bacilli. *Ann. rheum. Dis.*, **34**, 326–331.

FORD-HUTCHINSON, A.W., SMITH, M.J.H., ELLIOTT, P.N.C., BOLAM, J.P., WALKER, J.R., LOBO, A.A., BADCOCK, J.K., COLLEDGE, A.J. & BILLIMORIA, F.J. (1975). Effects of a human plasma fraction on leucocyte migration into inflammatory exudates. *J. Pharm. Pharmac.*, **27**, 106–112.

SAXENA, P.N. (1960). Effect of drugs on early inflammatory reactions. *Arch. int. Pharmacodyn. Ther.*, **126**, 228–237.

SWINGLE, K.F. (1974). Evaluation for anti-inflammatory activity. In *Anti-inflammatory agents, chemistry and pharmacology*. Vol. II, ed. Scherrer, R.A. & Whitehouse, M.W. pp. 33–122. New York: Academic Press.

WIENER, S., LENDVAI, S., ROGERS, B., URIVETSKY, M. & MEILMAN, E. (1973). Non-immune chemotaxis *in vivo*. *Am. J. Path.*, **73**, 807–816.

WIENER, S.L., WIENER, R., URIVETSKY, M., SHAFER, S., ISENBERG, H.D., JANOV, C. & MEILMAN, E. (1975). The mechanism of action of a single dose of methylprednisolone on acute inflammation *in vivo*. *J. clin. Invest.*, **56**, 679–689.

---

## Pethidine pharmacokinetics in dog: dose and time studies

M.R. BENDING, P.N. BENNETT & M. ROWLAND

*Department of Clinical Pharmacology, Royal Postgraduate Medical School, London and Department of Pharmacy, University of Manchester*

Data in man suggest that pethidine is highly cleared predominantly by metabolism and may therefore exhibit a 'first pass' effect on oral dosing (Mather, Tucker, Pflug, Lindop & Wilkerson, 1975; Chan, Kendall, Wells & Mitchard, 1975). To examine and extend this possibility, the effect of dose and time on the oral availability and disposition kinetics of pethidine were studied in three female greyhound dogs. In the dose-range study the following doses were administered on separate occasions—1.0, 2.0 and 3.0 mg/kg intravenously (i.v.) and 2.0, 3.0 and 4.0 mg/kg orally (p.o.) by stomach tube. In the time study, each dog received 2.0 mg/kg i.v. eight-hourly for seven doses and on another occasion 3.0 mg/kg p.o. eight-hourly for seven doses.

The terminal half-life of the blood-concentration-time curve increased with dose (means—50 and 72 min at the 1.0 and 3.0 mg/kg i.v. doses and 77 and 103 min at the 2.0 and 4.0 mg/kg p.o. doses). Comparison of the area under the blood-concentration-time curve (AUC) with dose suggested a non-linear relationship with AUC increasing dis-proportionately at higher doses. Consequently total systemic blood clearance of pethidine (dose i.v./AUC i.v.) fell with increasing dose, the values for the three dogs at the 1.0 and 3.0 mg/kg i.v. doses being 59 and 34, 51 and 42, 65 and 52 ml $\text{min}^{-1}$ $\text{kg}^{-1}$ respectively. However a plot of $\beta$ (the terminal disposition rate constant) × AUC against dose (i.v.) was linear, indicating that volume of distribution was a constant (values for each dog—4.2, 4.4 and 3.6 l/kg). This indicates that changes in clearance are reflected in changes in $\beta$. In two dogs a plot of $\beta \times$ AUC against dose (p.o.) was linear and the systemic availability of pethidine in these dogs, calculated as the ratio of the slopes of the p.o. and i.v. regression lines was 0.41 and 0.38. In the third dog $\beta \times$ AUC against dose was non-linear: thus availability changed with dose in this dog between 0.57 and 0.64 at the 2.0 and 3.0 mg/kg p.o. doses.

The findings in single dose administrations of pethidine were reproducible when further single doses were given. The clearance of pethidine in these animals approached liver blood flow and the low oral availability is compatible with significant 'first pass' hepatic metabolism of pethidine. In the repeated dose study for both i.v. and p.o. administration, no accumulation of pethidine occurred and the kinetics of pethidine did not change with time. The data suggest that while pethidine may exhibit dose-dependent disposition kinetics, there is no evidence for time dependent kinetics at the doses studied.

### References

CHAN, K., KENDALL, M.J., WELLS, W.D.E. & MITCHARD, M. (1975). Factors influencing the excretion and relative physiological availability of pethidine in man. *J. Pharm. Pharmac.*, **27**, 235–241.

MATHER, L.E., TUCKER, G.T. PFLUG, E.A., LINDOP, M.J. & WILKERSON, C. (1975). Meperidine kinetics in man. Intravenous injection in surgical patients and volunteers. *Clin. Pharmacol. Therap.*, **17**, 21–30.

---

## Fluorimetric determination of ampicillin and cephalexin

R.H. BARBHAIYA & P. TURNER

*Department of Clinical Pharmacology, St Bartholomew's Hospital, London EC1A 7BE*

A fluorimetric method for quantitative analysis of two $\beta$-lactam antibiotics ampicillin and cephalexin has been developed, based upon the production of fluorescent derivatives formed by alkaline hydrolysis with 2N NaOH and heating at 100°C. The rate of formation of fluorescent material is enhanced by addition of formaldehyde in the buffer. The fluorescent compounds exhibit uncorrected excitation and emission spectra at 345 and 420 nm respectively. The structural similarities between the side chains of ampicillin and cephalexin and their identical fluorescent spectra suggest that both the antibiotics could be forming a similar fluorescent derivative. Although the precise chemical identity of the fluorescent derivative has not yet been determined, there is evidence to suggest that it may be the diketopiperazine derivative (Cohen, Funke & Puar, 1973; Indelicato, Narvilas, Pfeiffer, Wheeler & Wilham, 1974, 1974; Yamana, Tsuji, Kanayama & Nakono, 1974). An assay procedure based on these observations permits detection of less than 0.1 μg/ml of ampicillin and cephalexin in aqueous solution and plasma. Because of greater sensitivity, the proposed method is more suitable for clinical studies of these antibiotics than the previously described acid hydrolysis method for ampicillin (Jusko, 1971).

The results obtained with the fluorimetric assay have been compared with the results obtained in microbiological assay. The fluorimetric assay has advantage over microbiological assay in terms of ease and speed of performance, reproducibility and sensitivity.

### References

COHEN, A.I., FUNKE, P.T. & PUAR, M.S. (1973). Alkaline degradation product of cephradine. *J. Pharm. Sci.*, **62**, 1559–1561.

INDELICATO, J.M., NARVILAS, T.T., PFEIFFER, R.R., WHEELER, W.J. & WILHAM, W.L. (1974). Substituent effect upon the base hydrolysis of penicillins and cephalosporins. *J. Med. Chem.*, **17**, 523–527.

JUSKO, W.J. (1971). Fluorometric analysis of ampicillin in biological fluids. *J. Pharm. Sci.*, **60**, 728–732.

YAMANA, T., TSUJI, A., KANAYAMA, K. & NAKONO, O. (1974). Comparative stabilities of cephalosporins in aqueous solutions. *J. Antibiot.*, **27**, 1000–1002.

## Phenylethylamine formation in perfused rat liver

K. BLAU, B. GOODWIN,
H.F. WOODS & M.B.H. YOUDIM

*Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London and MRC Clinical Pharmacology Unit, Radcliffe Infirmary, Oxford*

Phenylethylamine is a sympathomimetic amine which can be formed via decarboxylation of L-phenylalanine or derived from dietary constituents (Nakajima, Kakimoto & Sano, 1964; Sandler, Youdim & Hanington, 1974). This amine can pass the blood-brain barrier and has been implicated in the pathogenesis of certain affective disorders (Sandler & Reynolds, 1976). There is little quantitative information concerning the formation of phenylethylamine from L-phenylalanine and the aim of the present experiments was to determine the extent of its formation from L-phenylalanine in rat liver.

When livers from fed female Wistar rats were perfused (Hems, Ross, Berry & Krebs, 1966) with glucose (5 mmol/l) and L-phenylalanine (0–30 mmol/l), at amino acid concentrations above 5 mmol/l there was a substantial decarboxylation of L-phenylalanine (Youdim, Mitchell & Woods, 1975) measured by the accumulation of phenylacetic acid and mandelic acid. These acids which were identified and measured by gas chromatography and mass spectrometry (Goodwin, Ruthven & Sandler, 1974, 1975) accounted for 0.6% of the L-phenylalanine removed at initial concentrations below 5 mmol/l. This figure rose to 2.2% at 10 mmol/l and reached 8% above 20 mmol/l (Figure 1).

Phenylethylamine is a substrate for hepatic monoamine oxidase (MAO) 'type B' (Youdim, 1975) and thus these figures represent an under-estimate of phenylethylamine formation. Treatment of the rats with the MAO inhibitor tranylcypromine (20 mg/kg i.p.) 2 h before perfusion resulted in the accumulation of phenylethylamine. When the initial L-phenylalanine concentrations were 0, 1, 5 and 30 mmol/l the amounts of phenylethylamine formed in 2 h were 1.0, 1.72, 18.4 and 32.8 μg/g wet liver respectively. In the absence of tranylcypromine only trace amounts of phenylethylamine were detected. This fact, taken together with the accumulation of mandelic and phenylacetic acids under these conditions suggests that normally phenylethylamine is a transient intermediate.

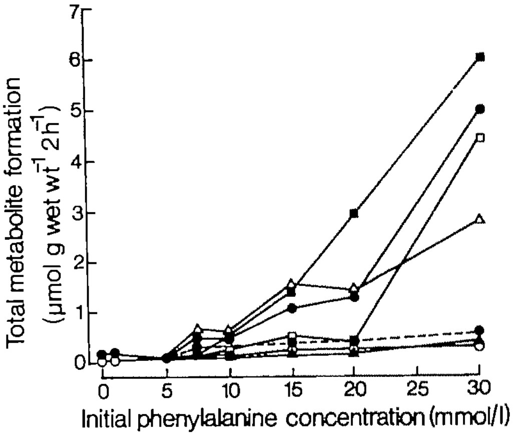


**Figure 1** Formation of metabolites from L-phenylalanine in the isolated perfused rat liver.

●——● Phenylpyruvic acid, ○——○ O-Hydroxyphenylacetic acid, □——□ Phenyl lactic acid, △——△ Phenylacetic acid, ■——■ Mandelic acid, ●– – –● *P*-Hydroxyphenyl lactic acid, ▲——▲ *P*-Hydroxyphenylacetic acid. The figures are means for between two and five estimations.

### References

GOODWIN, B.L., RUTHVEN, C.R.J. & SANDLER, M. (1974). Gas chromatographic estimation of urinary homovanillic and 4-hydroxy-3-methoxy mandelic acids using capillary columns. *Clin. Chim. Acta*, **44**, 111–112.

GOODWIN, B.L., RUTHVEN, C.R.J. & SANDLER, M. (1975). Gas chromatographic assay of phenylacetic acid in biological fluid. *Clin. Chim. Acta*, **62**, 443–446.

HEMS, R., ROSS, B.D., BERRY, M.N. & KREBS, H.A. (1966). Gluconeogenesis in the perfused rat liver. *Biochem. J.*, **101**, 284–292.

NAKAJIMA, T., KAKIMOTO, Y. & SANO, I. (1964). Formation of $\beta$-phenylethylamine in mammalian tissue and its effect on motor activity in the mouse. *J. Pharmac. exp. Ther.*, **143**, 319–325.

SANDLER, M. & REYNOLDS, G.P. (1976). Does phenylethylamine cause schizophrenia? *Lancet*, **i**, 70–71.

SANDLER, M., YOUDIM, M.B.H. & HANINGTON, E. (1974). A phenylethylamine oxidizing defect in migraine. *Nature (Lond.)*, **250**, 335–337.

YOUDIM, M.B.H. (1975). Monoamine oxidase: its inhibition. In *Modern Problems of Pharmacopsychiatry*, Vol. **10**, ed. Mendlewicz, J. pp. 65–88. Basel: Karger.

YOUDIM, M.B.H., MITCHELL, B. & WOODS, H.F. (1975). The effect of increasing phenylalanine concentration on phenylalanine metabolism in perfused rat liver. *Biochem. Soc. Trans.*, **3**, 683–684.

# The effect of glucocorticoids on turn-over of acetylcholine and prostaglandins in the isolated guinea-pig ileum

R.M. CHICHMANIAN, I. KINSUN,
M.A. MOULIN & E.C. SAVINI

*Departement de Pharmacologie, Chemin de Vallombrose, 06034 Nice, France*

According to Ferreira, Herman & Vane (1976), pieces of rabbit jejunum kept in Krebs solution are able to release prostaglandins $E_2$ and $F_{2\alpha}$ during their spontaneous contractions. These secretions were changed neither by contracting substances acetylcholine and physostigmine, nor by relaxing substances (adrenaline, hyoscine or papaverine). On the other hand, Botting & Salzmann (1974) showed that guinea-pig isolated gut also releases prostaglandins, and this secretion is inhibited by indomethacin. Finally, Bennett, Eley & Stockley (1975) brought strong evidence for the contracting effects of prostaglandins $E_1$, $E_2$, $F_{1\alpha}$ and $F_{2\alpha}$ on the longitudinal muscle of the guinea-pig ileum.

The aim of this preliminary investigation is to bring evidence for the inhibition of prostaglandins release in the organ bath fluid after administration of anti-inflammatory steroids: cortisol, prednisolone, methylprednisolone, dexamethasone, triamcinolone, betamethasone and fluorocortisol given at doses in a range of 0.1 to 1000 µg/ml). The steroids used as soluble esters (hemisuccinate, $Na^+$ phosphate) were introduced into the bath filled up with Krebs solution and bubbled with $CO_2+O_2$. In rare cases we noticed occasional small contractions at low concentrations with cortisol, prednisolone, methylprednisolone and dexamethasone, but in most of the cases even higher subsequent doses produced no contraction. Sensitivity to acetylcholine, histamine, prostaglandins $E_2$ and $F_{2\alpha}$ was not changed.

During coaxial stimulation (Paton, 1955) the height of the twitches was not reduced by prednisolone and fluorocortisol while indomethacin still decreased the height of the twitch.

In another batch of guinea-pigs reserpine (5 mg/kg) was injected one day before the experiment; the corresponding ileum did not contract even after large doses of glucocorticoids but during the action of hemicholinium (HC3, 120 µg/ml during 90 min), prednisolone and fluorocortisol were able to stimulate the ileum again probably because the synergism established between HC3 and corticosteroids as far as acetylcholine release is concerned.

$PGE_2$ and $PGF_{2\alpha}$ were measured by radioimmunoassay in a sample of liquid extracted from the organ bath. After gut contraction due to acetylcholine, an increase of $PGE_2$ release proportional to acetylcholine concentration is observed. Acetylcholine does not influence $PGF_{2\alpha}$ release. After coaxial stimulation of guinea-pig ileum, increase of $PGE_2$ release after 10 volleys of DC shocks (every minute) under similar conditions, fluorocortisol (1 mg/ml in the bath) reduces $PGE_2$ release.

## References

BENNETT, A., ELEY, K.G., STOCKLEY, H.L. (1975). The effects of prostaglandins on guinea-pig isolated intestine and their possible contribution to muscle activity and tone. *Br. J. Pharmac.*, **54**, 197–204.

BOTTING, J.H. & SALZMANN, R. (1974). The effect of indomethacin on the release of prostaglandin $E_2$ and acetylcholine from guinea-pig isolated ileum at rest and during field stimulation. *Br. J. Pharmac.*, **50**, 119–124.

FERREIRA, S.H., HERMAN, A.G. & VANE, J.R. (1976). Prostaglandin production by rabbit isolated jejunum and its relationship to the inherent tone of the preparation. *Br. J. Pharm.*, **56**, 469–477.

PATON, W.D.M. (1955). The response of the guinea-pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol. (Lond.)*, **127**, 40–41P.

# INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac,* **50**, 3-23, may also be obtained. Specific points to note are:

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors **in alphabetical order**. A short title of not more than 50 letters should also be suggested.

2. **Statement** Papers are accepted only if accompanied by a statement that they have not been and will not be published in whole or in part in any other Journal.

3. **Summary** (2 copies) This should be a short summary of results and conclusions arranged in numbered paragraphs.

4. **Abstract** (2 copies) An abstract of about 100 words suitable for inclusion in an abstracting journal should be typed on a separate sheet, giving the author's name and address and the title of the paper.

5. **Figures** These should not be larger than A4 (206 x 294 mm) and may be in the form of original drawings, recorded tracings or high quality photographic prints made from them. Negative prints of kymograph tracings (black on white) should be provided, and both ordinates and abscissae should be calibrated. Lettering on figures should be inserted in pencil. Symbols which are to appear in figures or legends should be chosen from the following:

○ ● □ ■ △ ▲ ▽ ▼

Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction.

6. **Tables** These should not have more than 85 characters to a line (counting spaces between columns as 4 characters) and certainly not more than 110 characters to a line, unless absolutely unavoidable. **Figure legends** and **tables** should be typed on separate sheets of paper.

7. **References** These should be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the fourth edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should in addition, include the names of editors, the edition number, where appropriate, and the town of origin and name of publisher.

**Two page proofs** will be supplied, one of which may be retained by the authors. The other should be corrected immediately and returned to the Press Editor. Corrections should be kept to a minimum.

**Twenty-five reprints** will be supplied to the authors free of charge. Additional reprints can be purchased. A reprint order form, which should be returned promptly, will be sent out with the proofs. No reprints of abstracts are supplied.

**Short Communications** These may be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text and only one figure or table. The summary should be a single paragraph. An abstract and statement should be included.

# British Journal of Pharmacology

VOLUME 58 NUMBER 3 NOVEMBER 1976

323 CLARK, A.L. & MITCHELSON, F. The inhibitory effect of gallamine on muscarinic receptors

333 EAKINS, K.E., RAJADHYAKSHA, V. & SCHROER, R. Prostaglandin antagonism by sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl]phenyl phosphonate (N-0164)

341 SEGAL, M. Glutamate antagonists in rat hippocampus

347 RICHARDS, C.D. & SMAJE, J.C. Anaesthetics depress the sensitivity of cortical neurones to L-glutamate

359 SMAJE, J.C. General anaesthetics and the acetylcholine-sensitivity of cortical neurones

367 KRUK, Z.L. & ZARRINDAST, M.R. Mazindol anorexia is mediated by activation of dopaminergic mechanisms

373 BISCOE, T.J., EVANS, R.H., HEADLEY, P.M., MARTIN, M.R. & WATKINS, J.C. Structure-activity relations of excitatory amino acids on frog and rat spinal neurones

383 DEY, P.K. & FELDBERG, W. Analgesia produced by morphine when acting from the liquor space

395 BARTLET, A.L. Actions of carbon tetrachloride, hexachloroethane and the products of their metabolism in sheep on *Fasciola hepatica*

401 HENNINGSSON, S. & ROSENGREN, ELSA. The effect of nandrolone, an anabolic steroid on putrescine metabolism in the mouse

407P PROCEEDINGS OF THE BRITISH AND FRENCH PHARMACOLOGICAL SOCIETIES, The University of Oxford, 15–17 September, 1976

Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge

Volume 58 (4) pages [illegible]
ISSN 0007-1 88

24 ✓

# British Journal of Pharmacology

NOT TO BE LENT OUT

December 1976

:URRENT ISSUE

15.3.77

BRITISH HIGH COMMISSION

# Edited for the British Pharmacological Society by

A.W. Cuthbert *(Chairman)* H.M. Adam *(Secretary)*
Margaret Day *(Press Editor)*
J.D. Stephenson *(Assistant Press Editor)*

A.M. Barrett
W.G. Bisset
T.B. Bolton
D.A. Brown
B.A. Callingham
T.J. Crow
F. de Matteis
C.T. Dollery
V. Eisen
E.W. Gill
A.F. Green
D.F. Hawkins
Margarethe Holzbauer
D.B. Hope
J.S. Kelly
G.M. Lees
G.P. Lewis
I.H.M. Main
E.K. Matthews
J.F. Mitchell
P.A. Nasmyth
M.W. Parkes
M. Sandler
A.P. Silverman
M.A. Simmonds
F.M. Sullivan
A. Ungar
R.A. Webster

---

Papers will be considered for publication on all aspects of drug action, including chemotherapy.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ. Authors should consult the instructions inside the back cover.

*The British Journal of Pharmacology* is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription £45.00 (USA & Canada $99. Rest of world $92). (Orders must be accompanied by remittance. Prices correct at time of publication.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

Enquiries concerning advertising space and rates should be addressed to: Advertisement Department, Macmillan Journals Limited, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Copyright © 1976 by the *British Journal of Pharmacology.* All rights of reproduction are reserved in respect of all papers, articles, illustrations, etc., published in this journal in all countries of the world.

Second class postage paid at New York, N.Y.
US Mailing Agent, Expeditors of the Printed Word, Ltd,
527 Madison Avenue, New York, NY 10022.

# HEINEMANN

***Important New Books from Heinemann Medical***

**ADVERSE REACTIONS TO DRUGS** Second Edition

**Q.L. Wade** MA(Cantab), MD, FRCP(Lond), HonFRCPI, and **Linda Beeley** MA(Oxon), MRCP,

The second edition of this popular paperback has been completely revised as is necessary with a subject which has developed so rapidly in the last few years.

The authors give a short, concise and readable account of adverse drug interactions and their mechanisms; there are also chapters on the use of drugs in pregnancy and on drug reactions that concern dental surgeons.

Many besides doctors and dental surgeons will, however, find this book of interest. There is a fascinating discussion on the work of the pharmaceutical industry before a drug is used on man, on the problems that face those who carry out the first studies with a new drug in man and on the problems of drug regulatory bodies, such as the Medicines Commission and the American F.D.A. who are responsible for the safety of drugs that are marketed.

240 pages £3.20 net

**AN INTRODUCTION TO MECHANISMS IN PHARMACOLOGY AND THERAPEUTICS**

**H. Rogers** MA, MB, BCh, PhD, MRCP, and **R. Spector** MD, PhD, FRCP,
With Contributions by J. Trounce MD, FRCP,

This book, written by three leading men in their field, covers a range of topics—from the molecular aspects of drug-cell interactions to the principles underlying their use in the patient.

Pharmacology stands at a meeting point of several sciences, including Physiology, Biochemistry, Cellular Biology and Clinical Medicine. The main theme of the book is to link some of the knowledge about the molecular aspects of drug action, and the treatment of disease in the clinical situation. Research and therapy with drugs today relies increasingly on an understanding of fundamental biological processes, and the approach which the authors present stresses the relevance of pre-clinical sciences to therapeutic practice.

428 pages £7.50 net

**William Heinemann Medical Books Ltd**
23 Bedford Square, London WC1B 3HT

# British Journal of Pharmacology

British Journal of Pharmacology provides up-to-date research on all aspects of drug action essential to those involved in pharmacology. Ensure you receive this information each month by taking a subscription.

Subscription Dept, Macmillan Journals Ltd, Brunel Road, Basingstoke, Hants RG21 2XS, England.

Please enter me for a year's subscription to the BRITISH JOURNAL OF PHARMACOLOGY starting with the issue dated . . . . . . . . . . . .

I enclose payment of £45.00 (USA & Canada $99. Rest of world $92).

Prices correct at time of publication. Payment may be made in any currency at the current exchange rates. Orders can only be processed if accompanied by the correct remittance. Cheques should be made payable to Macmillan Journals Ltd.

Name . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Address . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Registered No: 785998 England Registered Office: 4 Little Essex St, London WC2R 3LF

# British Journal of Clinical Pharmacology

**THE BRITISH JOURNAL OF CLINICAL PHARMACOLOGY** provides a forum for the exchange of information and ideas between the pharmaceutical industry and the medical profession. First published just over two years ago, the BJCP has in this very short time become the most authoritative channel of communication between these two areas of research and practice in clinical pharmacology. By presenting the latest findings of new research, while keeping the ultimate object of patient care always in mind, the BJCP acts as a vitally needed educator in this fast-moving area of science.

BJCP contains papers and reports on all aspects of drug action in man, in both health and in disease. These include the pre-clinical testing of drugs in normal volunteers and important clinical trials in patients. Discussions often clarify the relationships between different drug therapies, and between drug therapies and non-drug therapies; the latter tend to be rather neglected in therapeutics. Particular attention is paid to advances in techniques, the methodology of study and investigations of more scrupulous clinical practice.

The editors contribute informed comment and evaluation of papers in terms of contemporary clinical practice, while letters and short communications allow immediate discussion of urgent observations and negative reports.

**THE BRITISH JOURNAL OF CLINICAL PHARMACOLOGY** is as fast-moving as the area of science it examines. Its expanding international readership testifies to its effectiveness as a vehicle of information and communication. Why not join the ever growing number of clinical pharmacologists all over the world who rely on the BJCP as their professional journal?

---

**Subscription Order Form**

To: Subscription Dept., Macmillan Journals,
Brunel Road, Basingstoke RG21 2XS, Hampshire, England.
Please enter me for a year subscription to the
**British Journal of Clinical Pharmacology.**
**Annual Subscription £28·00 (U.S.A. and Canada U.S.$65, Rest of World U.S.$60)**
(Payment must accompany order; cheques should be made payable to Macmillan Journals Limited; payment may be made in any currency at the current exchange rate.)

I enclose..................................................................................................

Name......................................................................................................

Address..................................................................................................

..............................................................................................................

**Prices correct at time of publication**

**Registered No. 785998. Registered Office: 4 Little Essex Street, London WC2R 3LF.**

VOLUME 58(4) DECEMBER 1976

# British Journal of Pharmacology

479 PERSSON, C.G.A. Inhibitory innervation of cat sphincter of Oddi

483 AZUMA, H., BANNO, K. & YOSHIMURA, T. Pharmacological properties of N-(3′,4′-dimethoxycinnamoyl) anthranilic acid (N-5′), a new anti-atopic agent

489 METCALF, G. & MYERS, R.D. A comparison between the hypothermia induced by intraventricular injections of thyrotropin releasing hormone, noradrenaline or calcium ions in unanaesthetized cats

497 GIACHETTI, A. & HOLLENBECK, R.A. Extravesicular binding of noradrenaline and guanethidine in the adrenergic neurones of the rat heart: a proposed site of action of adrenergic neurone blocking agents

505 DAY, M.D. & ROACH, A.G. Cardiovascular effects of dopamine after central administration into conscious cats

517 CHEN, C.S., GATES, G.R. & REYNOLDSON, J.A. Effect of morphine and naloxone on priming-induced audiogenic seizures in BALB/c mice

521 ROSS, S.B. Long-term effects of *N*-2-chloroethyl-*N*-ethyl-2-bromobenzylamine hydrochloride on noradrenergic neurones in the rat brain and heart

529 WONG, P.Y.D. & YEUNG, C.H. Inhibition by amiloride of sodium-dependent fluid reabsorption in the rat isolated caudal epididymis

533 CURZON, G. & FERNANDO, J.C.R. Effect of aminophylline on tryptophan and other aromatic amino acids in plasma, brain and other tissues and on brain 5-hydroxytryptamine metabolism

547 PARRATT, J.R. & STURGESS, R.M. The effect of a new anti-inflammatory drug, flurbiprofen, on the respiratory, haemodynamic and metabolic responses to *E. coli* endotoxin shock in the cat

553 STEINESS, EVA & VALENTIN, N. Myocardial uptake of digoxin in chronically digitalized dogs

561 BROWN, F. & REDFERN, P.H. Studies on the mechanism of action of amantadine

569 COTMAN, C.W., HAYCOCK, J.W. & LEVY, W.B. On the functional coupling of neurotransmitter uptake and release in brain

573 McDONALD-GIBSON, WENDY J., SAEED, S.A. & SCHNEIDER, C. The local antinociceptive and topical anti-inflammatroy effects of propyl gallate in rodents

583 CONSTANTI, A. & NISTRI, A. Antagonism by some antihistamines of the amino acid-evoked responses recorded from the lobster muscle fibre and the frog spinal cord

593 DE JONG, W. & NIJKAMP, F.P. Centrally induced hypotension and bradycardia after administration of $\alpha$-methylnoradrenaline into the area of the nucleus tractus solitarii of the rat

599 HEDQVIST, P. Further evidence that prostaglandins inhibit the release of noradrenaline from adrenergic nerve terminals by restriction of availability of calcium

605 FAST, D. & TENENHOUSE, A. The effect of dibutyryl cyclic adenosine-3′,5′-monophosphate on protein secretion from the rat exocrine pancreas *in vitro*

613 BARLOW, R.B., BERRY, K.J., GLENTON, P.A.M., NIKOLAOU, N.M. & SOH, K.S. A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and in ileum at 29°C and 37°C

*Br. J. Pharmac.* (1976), **58**, 479–482

# INHIBITORY INNERVATION OF CAT SPHINCTER OF ODDI

C.G.A. PERSSON
AB Draco, Research and Development Laboratories, Fack, S-221 01 Lund, Sweden

**1** Electrical stimulation with trains of 0.1–0.2 ms pulses of the cat isolated sphincter of Oddi inhibited the spontaneous contractile activity and lowered base-line tension considerably. A contraction usually followed the period of stimulation.

**2** These inhibitory effects were prevented by tetrodotoxin 0.1–0.5 μg/ml but were not reduced by hexamethonium, morphine, or blockade of $\alpha$- or $\beta$-adrenoceptors or cholinoceptors with phenoxybenzamine propranolol or atropine, respectively.

**3** Adenosine-5′-triphosphate (ATP) and adenosine-5′-diphosphate (ADP) inhibited the spontaneous sphincter activity and caused relaxation thus mimicking the effects of the C-terminal octapeptide of cholecystokinin (C8-CCK), isoprenaline and prostaglandins $E_1$ and $E_2$.

**4** ATP alone (> 100 μg/ml) or ATP (> 10 μg/ml) plus dipyridamole (1 μg/ml), relaxed the sphincter to the same degree as did the field stimulation.

**5** In sphincters maximally contracted by acetylcholine, the effect of stimulation was more marked than that recorded in uncontracted preparations.

**6** The present findings suggest that the sphincter of Oddi receives inhibitory nerves that are neither cholinergic nor adrenergic.

## Introduction

The activity of the sphincter of Oddi might be controlled by several mechanisms (Hallenbeck, 1967; Persson, 1973; Andersson, Andersson, Hedner & Persson, 1973). The most important is considered to be the hormone cholecystokinin, which inhibits sphincter activity and tone without involving nerves (Persson, 1973). The role of the nerves is not settled. In the cat sphincter of Oddi the adrenergic innervation may counteract the effect of cholecystokinin, as stimulation of adrenergic nerves and administration of the transmitter amines contract the sphincter and increase its activity (Persson, 1973). Less is known about the cholinergic control, but exogenous acetylcholine contracts the sphincter of Oddi in most species (Hallenbeck, 1967).

Studies of gastro-intestinal motility have repeatedly demonstrated the presence of non-adrenergic inhibitory and non-cholinergic excitatory nerves (Bortoff, 1972) and the non-adrenergic nerves may be purinergic (see review by Burnstock, 1972).

The sphincter of Oddi may receive an innervation that is neither cholinergic nor adrenergic (Persson, 1971b), a suggestion which is further supported by the present experiments.

## Methods

Fourteen adult cats, fasted for 24 h but allowed water, were anaesthetized with sodium pentobarbitone (Abbott) (40 mg/kg i.p.) and bled out. The sphincter of Oddi was dissected free from surrounding duodenum and the bile duct was cut off. Without occluding the orifices, the sphincter was mounted as a longitudinal preparation in an organ bath, one end being connected to a strain-gauge transducer (Persson, 1971a). The bath contained Krebs solution with the following composition (mM): NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.15, $NaHCO_3$ 24.9, $KH_2PO_4$ 1.15 and glucose 5.5; pH was 7.4. It was kept at 37°C and gassed with a mixture of 95% $O_2$ and 5% $CO_2$. Changes in isometric longitudinal tension of the sphincter were recorded on a Grass Polygraph by means of strain-gauge transducers (FTO3).

Two platinum electrodes connected to an electronic stimulator (Grass S48 Stimulator) were fixed vertically, approximately 5 mm apart on opposite sides of the sphincter preparation. The preparation was stimulated at supra-maximal voltage (40 V) with trains of rectangular pulses delivered at regular intervals (usually 100 seconds). After preliminary trials at 0.05–0.5 ms, the pulse width was fixed at 0.1 ms and 5 Hz for most experiments.

The drugs used were: acetylcholine chloride (Calbiochem, U.S.A.), adenosine-5′-diphosphate (disodium salt) (Sigma Chemical Company, U.S.A.), adenosine-5′-triphosphate (disodium salt) (Sigma Chemical Company, U.S.A.), C-terminal octapeptide of cholecystokinin (C8-CCK) (Squibb, U.S.A.), dipyridamole (Boehringer Ingelheim, Germany), hexamethonium bromide (May & Baker Ltd.), (±)-isoprenaline chloride (Sigma Chemical Company, U.S.A.), morphine chloride (Pharmacopoea Nordica) (morphine hydrochloride), (−)-noradrenaline bitartrate (Sigma Chemical Company, U.S.A.), phenoxybenzamine hydrochloride (Smith, Kline & French), propranolol hydrochloride (ICI Ltd.), tetrodotoxin (Sankyo, Japan). The drugs were dissolved in fresh glass-distilled water before each experiment. Solutions of isoprenaline and noradrenaline contained ascorbic acid (0.2 mg/ml).

## Results

The sphincters exhibited spontaneous rhythmic contractile activity. Electrical stimulation inhibited the contractile activity and, except in two preparations, greatly lowered the base-line tension (Figures 1, 2, 4 and 5). In two preparations the response to stimulation was recorded as only an inhibition of the rhythmic activity. In seven preparations the period of stimulation was followed by contraction (Figure 1). The pulses were of 0.1 or 0.2 ms duration. Above 0.3 ms some of the response remained after tetrodotoxin (0.1–0.5 µg/ml) had completely inhibited the response to 0.2 ms pulses. Tetrodotoxin (TTX) (0.5 µg/ml) was fully effective 5 min after its addition to the bath, preventing the inhibitory effects and post-stimulus excitation of the sphincter. After washout of TTX, the response to electrical stimulation gradually recovered (Figure 2). As reported previously (Persson, 1971b), TTX by itself, in some preparations, produced a stimulant effect which showed tachyphylaxis.

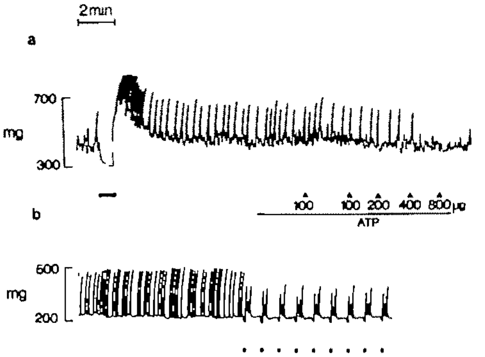


Figure 1 (a) Effect of electrical stimulation and adenosine triphosphate (ATP) on spontaneous contractions of the sphincter of Oddi. At bar, electrical stimulation for 40 s inhibited contractile activity. A marked excitation occurred after the stimulus ended. ATP added cumulatively to the bath (50 ml) inhibited the spontaneous activity. (b) Effect of electrical stimulation (dots) at regular intervals on spontaneous activity of a sphincter of Oddi. Sphincter tone was reduced; note also changes in the pattern of contractile activity.

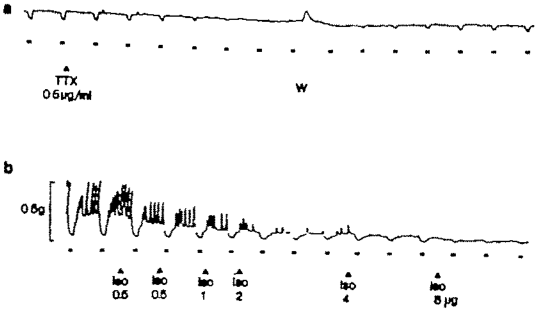


Figure 2 (a) Tetrodotoxin (TTX) inhibited the effect of field stimulation (bars) in cat isolated sphincter of Oddi without spontaneous contractile activity. After washing (W), the relaxant effect gradually returned. Due to tachyphylaxis no excitatory effect of TTX can be seen. (b) Isoprenaline (ISO) added cumulatively to the bath (50 ml) relaxed the sphincter; high concentrations relaxed the sphincter to the same extent as with field stimulation (bars).

The inhibitory response to electrical stimulation was not changed by atropine (0.1 µg/ml), phenoxybenzamine (0.1 µg/ml) or propranolol (0.1 µg/ml); the antagonists were used in concentrations which prevented, respectively, contractions induced by acetylcholine (0.1 µg/ml) and noradrenaline (0.1 µg/ml) or relaxations due to isoprenaline (0.2 µg/ml). Hexamethonium (100 µg/ml) was also without effect, as was morphine (0.1–10 µg/ml); a range of frequencies between 1 and 5 Hz was tried in the presence of morphine. The blocking agents were left in contact with the sphincter preparations for up to 20 minutes.

ATP (2–30 µg/ml) or adenosine-5′-diphosphate (ADP, 4–30 µg/ml) inhibited spontaneous activity of the sphincter but the basal tension was only slightly

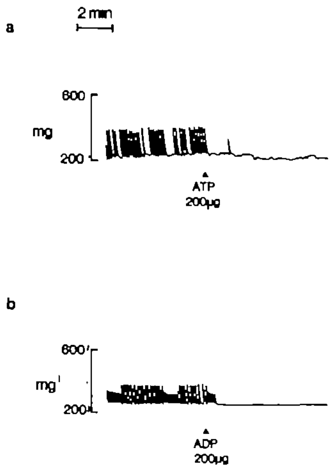


**Figure 3** (a) Inhibition of contractile activity in the sphincter of Oddi by adenosine triphosphate (ATP). (b) Inhibitory effect of ATP mimicked by ADP. Bath volume, 50 ml.

lowered (Figures 3 and 4). In concentrations above 100 µg/ml, ATP produced a relaxation as pronounced as that induced by electrical stimulation (Figure 4). In the presence of dipyridamole, 1 µg/ml, a pronounced relaxation was seen at lower concentrations of ATP (approx. 10 µg/ml) (Figure 4); dipyridamole alone did not change the response to field stimulation. Prostaglandins $E_1$ and $E_2$, C8-CCK and isoprenaline also inhibited the contractile activity, and lowered the base-line tension (cf. Persson, 1973; Andersson *et al.*, 1973), producing a maximum equalled by that obtained with field stimulation (Figures 2 and 5).

When the sphincter was maximally contracted by acetylcholine (1 µg/ml), the effect of electrical stimulation was more marked than that recorded in uncontracted preparations (Figure 5). In the presence of ACh (1 µg/ml) ATP at a given concentration relaxed the sphincters less than it did when they were uncontracted. In addition, ATP up to 1 mg/ml in contracted preparations did not produce a relaxation as pronounced as that induced by electrical stimulation. The post-stimulation excitatory responses recorded in seven of the fourteen sphincters (Figure 1) were not inhibited by hexamethonium (100 µg/ml) or indomethacin (1–4 µg/ml), and they were only slightly diminished by atropine (0.1 µg/ml) or phenoxybenzamine (0.1 µg/ml). The size of the post-stimulus response gradually diminished during the experiment and in five preparations had vanished after approximately 3 h while the inhibitory effect remained intact. Some preparations lost spontaneous activity after 1–3 h and an inhibitory effect was seen only as a lowering of base-line tension (Figure 2).

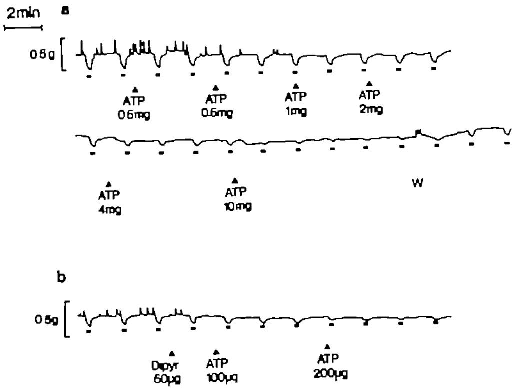


**Figure 4** (a) Adenosine triphosphate (ATP) added cumulatively to the bath gradually relaxed the isolated sphincter of Oddi to the same extent as field stimulation (bars). (b) Potentiation of effect of ATP by dipyridamole (Dipyr.). Electrical stimulation at bars. Bath volume, 50 ml.

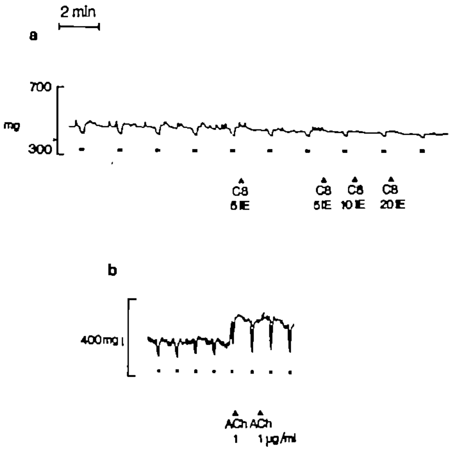


**Figure 5** (a) Reduction in tone of isolated sphincter of Oddi and response to electrical stimulation by octapeptide of cholecystokinin (C8); IE = Ivy dog units of cholecystokinin. Bath volume, 50 ml. (b) Pronounced lowering of sphincter tone by electric stimulation (bars). When the sphincter was maximally contracted by acetylcholine (ACh) the relaxation was even more marked.

## Discussion

Responses of the isolated sphincter of Oddi preparation agree well with those obtained *in vivo* and the changes in longitudinal tension (oblique and longitudinal muscle fibres) closely reflect changes in resistance to flow through the sphincter (Persson, 1973). Resistance of the sphincter is influenced by the intrinsic nerves, which are perhaps predominantly inhibitory. Although high concentrations were required to produce a relaxation as great as that induced with electrical stimulation, ATP and ADP were found to relax the sphincter, a finding which suggests that 'purinergic' nerves may be involved (see Burnstock, 1972). It has been shown that ATP alone does not mimick the neural response but does so in the presence of dipyridamole (Coleman & Levy, 1974). In the present study, dipyridamole diminished the concentration of ATP needed to produce inhibitory effects; this potentiation may have been due to the uptake of adenosine being blocked by dipyridamole.

Sphincter relaxation was also produced by isoprenaline, acting on $\beta$-adrenoceptors, by the octapeptide of cholecystokinin, and by prostaglandins $E_1$ and $E_2$. These effects have also been shown previously (Persson, 1971a; 1973; Andersson *et al.*, 1973). However, since the effects of cholecystokinin, prostaglandins, ATP and ADP cannot be selectively antagonized, their possible participation in the inhibitory effect of field stimulation is difficult to investigate.

A cholinergic or adrenergic component of post-stimulus contractions cannot be excluded nor assessed quantitatively due to the development of tachphylaxis. The contractions may have been myogenic in origin, as suggested by Furness (1971) for the guinea-pig intestine but this is unlikely since they were prevented by TTX. On the other hand, the phenomenon might be due to increased prostaglandin synthesis as recently reported for intestinal muscle by Burnstock, Cocks, Paddle & Staszewska-Barczak (1975). This explanation is also not plausible since prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ all inhibit the isolated sphincter of Oddi (Andersson *et al.*, 1973).

Persson (1971b) found that TTX apparently stimulated the isolated sphincter of Oddi; this did not seem to be due to inhibition of cholinergic or adrenergic nerves. This effect of TTX, which was confirmed in the present experiments, may result from inhibition of conduction in the non-adrenergic, possibly 'purinergic', inhibitory nerves.

The expert technical assistance of Mrs Marianne Ekman is gratefully acknowledged.

## References

ANDERSSON, K.E., ANDERSSON, R., HEDNER, P. & PERSSON, C.G.A. (1973). Parallelism between mechanical and metabolic responses to cholecystokinin and prostaglandin $E_2$ in extra-hepatic biliary tract. *Acta physiol. scand.*, **89**, 571–579.

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmac. Rev.*, **24**, 509–581.

BURNSTOCK, G., COCKS, T., PADDLE, B. & STASZEWSKA-BARCZAK, J. (1975). Evidence that prostaglandin is responsible for the 'rebound contraction' following stimulation of non-adrenergic, non-cholinergic ('purinergic') inhibitory nerves. *Eur. J. Pharmac.*, **31**, 360–362.

BORTOFF, A. (1972). Digestion: Motility. *A. Rev. Physiol.*, **34**, 261–290.

COLEMAN, R.A. & LEVY, G.P. (1974). A non-adrenergic inhibitory nervous pathway in guinea-pig trachea. *Br. J. Pharmac.*, **52**, 167–174.

FURNESS, J.B. (1971). Secondary excitation of intestinal smooth muscle. *Br. J. Pharmac.*, **41**, 213–226.

HALLENBECK, G.A. (1967). Biliary and pancreatic intraductal pressures. In *Handbook of Physiology: Alimentary Canal.* ed. Heidel, W. pp. 1007–1026. Baltimore: Williams and Wilkins Co.

PERSSON, C.G.A. (1971a). Adrenoceptor functions in the cat choledochoduodenal junction *in vitro*. *Br. J. Pharmac.*, **42**, 447–461.

PERSSON, C.G.A. (1971b). Excitatory effect of tetrodotoxin in an isolated smooth muscle organ. *J. Pharm. Pharmac.*, **23**, 986–987.

PERSSON, C.G.A. (1973). Adrenergic, cholecystokinetic and morphine-induced effects on extra-hepatic biliary motility. *Acta physiol. scand. Suppl.* **383**.

*(Received December 22, 1975.*
*Revised April 10, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 483–488

# PHARMACOLOGICAL PROPERTIES OF N-(3′,4′-DIMETHOXYCINNAMOYL) ANTHRANILIC ACID (N-5′), A NEW ANTI-ATOPIC AGENT

H. AZUMA, K. BANNO & T. YOSHIMURA
Division of Pharmacology, Research Laboratories, Kissei Pharmaceutical Co. Ltd.,
Matsumoto City 399-65, Japan

**1** N-(3′,4′-dimethoxycinnamoyl) anthranilic acid (N-5′) exhibited a dose-dependent, potent inhibition of the passive cutaneous anaphylaxis (PCA) mediated by homocytotropic antibodies (HTA), which was hardly affected by anti-inflammatory agents such as phenylbutazone, indomethacin and prednisolone at any dose used. The HTA-induced PCA was significantly inhibited by combined treatment with diphenhydramine and cyproheptadine.

**2** Doses of N-5′ which potently inhibited HTA-induced PCA inhibited only slightly the heterologous PCA produced by anti-bovine serum albumin (BSA) rabbit serum. This heterologous PCA was clearly inhibited by phenylbutazone, indomethacin and prednisolone. Diphenhydramine and cyproheptadine, singly or combined inhibited the heterologous PCA only slightly.

**3** The increased vascular permeability caused by histamine and 5-hydroxytryptamine was significantly inhibited by diphenhydramine or cyproheptadine, but not by N-5′ and the anti-inflammatory agents used.

**4** N-5′ 150 mg/kg orally inhibited rat paw oedema induced by carrageenin by about 26% while phenylbutazone, indomethacin and prednisolone produced significant inhibition.

**5** N-5′ at concentrations of 100 and 1000 µM significantly inhibited (by about 52% and 95%, respectively) the histamine release from rat peritoneal cells induced by HTA; 10 µM N-5′ had little effect. Histamine release was inhibited by phenylbutazone or indomethacin at 1000 µM but not at 100 µM. Prednisolone had no effect on histamine release at any of the concentrations used.

**6** These findings suggest that the inhibition of the HTA-induced PCA by N-5′ may be due to inhibition of histamine release and is clearly different from the actions of anti-inflammatory agents such as phenylbutazone, indomethacin and prednisolone.

## Introduction

N-(3′,4′-dimethoxycinnamoyl) anthranilic acid (N-5′) is a newly synthesized, anthranilic acid derivative. Given orally, N-5′ potently inhibits the passive cutaneous anaphylaxis (PCA) mediated by homocytotropic antibodies (HTA).

Koda, Nagai, Watanabe, Yanagihara & Sakamoto (1976) reported that N-5′ inhibited HTA-induced PCA and mast cell degranulation in rats and guinea-pigs. They suggested that the anti-allergic properties of N-5′ may be clinically useful in the treatment of allergy-related diseases, especially asthma.

In view of the anti-inflammatory actions of anthranilic acid derivatives, N-5′ has been compared with several established anti-inflammatory agents.

## Methods

### *Passive cutaneous anaphylaxis in rats*

*Preparation of antisera.* Antiserum containing homocytotropic antibody (HTA, reagin antibody) was prepared in rats as described by Tada & Okumura (1971). Male Wistar rats weighing 180 to 200 g were splenectomized and 5 days later immunized by injecting into all four footpads a total of 1 mg of 2,4-dinitrophenyl-coupled ascaris extract (DNP-As) mixed with $10^{10}$ *Bordetella pertussis*; 5 days later, 0.5 mg of DNP-As alone was injected subcutaneously into the back. Eight days after the first immunization, blood was collected by aortic puncture, under ether anaesthesia. The antiserum obtained by these procedures remained, when injected intradermally, in the skin for at least 1 week and was inactivated by heating at 56°C for 30 minutes. The titre of the antiserum was determined in rats by homologous PCA 48 h after injecting it intradermally. The dilution of antiserum producing a diameter of approximately 5 mm was usually 1:500. Anti-bovine serum albumin (BSA) rabbit serum (anti-BSARS) was prepared by 4 weekly intramuscular injections of BSA (10 mg/kg) emulsified with an equal volume of complete Freund's adjuvant. Anti-BSARS injected intradermally

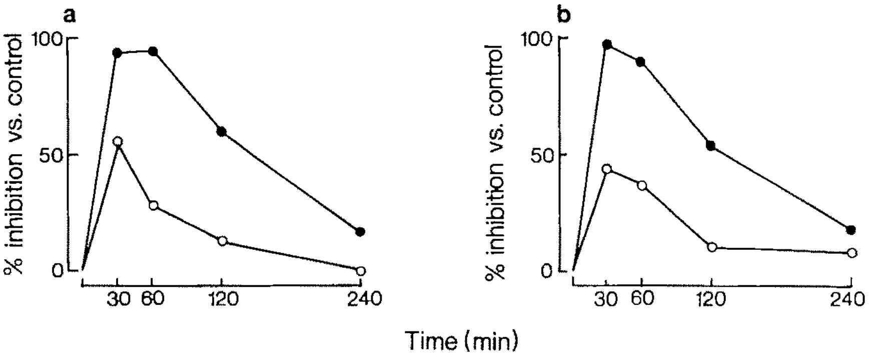


**Figure 1** Time course of inhibitory action of N-5′ on (a) the amount of leakage of dye and (b) the area resulting from passive cutaneous anaphylaxis produced by homocytotropic antibodies. N-5′ 100 (○) or 200 (●) mg/kg was administered orally at 30, 60, 120 or 240 min before challenge with antigen (DNP-As). Each point represents the mean value of 6 observations.

disappeared within 24 h and the titre determined by 4 h-PCA was 1 : 32. All antisera obtained were stored at −20°C until used.

*Assessment of passive cutaneous anaphylaxis in rats.* Normal male Wistar rats weighing 140 to 160 g were sensitized passively by injection into the shaved skin of the diluted antisera (1 : 200 in the case of HTA, 1 : 4 in the case of anti-BSARS). After 48 h in the case of HTA and 4 h in the case of anti-BSARS, 0.5 mg of antigen (DNP-As or BSA, respectively) and 0.5 ml of 0.5% Evans blue were injected intravenously. The animals were exsanguinated 30 min after challenge with the antigens. The area blued as a result of PCA was excised and measured. The amount of dye leaked was extracted with acetone containing sodium sulphate and measured photometrically (620 nm). The drugs to be tested were suspended in a 0.5% aqueous solution of sodium carboxymethylcellulose (CMC) and administered orally except where stated otherwise. Animals were fasted for 16 h before experiments and water was provided *ad libitum.*

The increase in vascular permeability caused by intradermal injections of histamine (10 μg/0.1 ml) or 5-hydroxytryptamine (0.1 μg/0.1 ml) was also assessed by the leakage of Evans blue.

### *Oedema of rat hind paw*

Oedema was induced in male Wistar rats (weighing 120 to 140 g and fasted for 16 h) by subcutaneous injection of 1.0% carrageenin (0.1 ml) into the hind paw. Drugs to be tested were administered orally 30 min before the injection of carrageenin. The oedema was measured volumetrically every hour for 5 h and expressed as (Et − En/En) × 100, where En = volume of hind paw before injection of carrageenin, and Et = volume after injection.

### *Anaphylactic histamine release from rat peritoneal cells* in vitro

*Isolation and sensitization of peritoneal cells.* Male Wistar rats weighing 180 to 220 g were exsanguinated and injected intraperitoneally with 10 ml of a solution containing (mmol/l): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2.6H_2O$ 1.0 and glucose 5.6; 5 units/ml of heparin was added and the pH adjusted to 7.2 with 5% (v/v) of 0.1 M phosphate buffer. The abdominal region was massaged for several minutes, and the peritoneal exudate then collected into siliconized glassware.

The cells were washed with the buffer solution and centrifuged (200 *g* for 3 min at 4°C) several times, and then separated. Cells from 10 animals were pooled. Peritoneal cells obtained in this way contained 34 to 35% mast cells.

The cells were sensitized by incubation, with occasional stirring, for 1 h at 37°C in 4 ml of homocytotropic antiserum diluted with 0.9% w/v NaCl solution (saline) and containing 10 units of heparin. They were then washed twice with the buffer solution. Finally a suspension of about $6 \times 10^4$ mast cells/ml was prepared.

*Histamine release and its assay.* After pre-warming to 37°C for 5 min, 2.5 ml of the cell suspension was incubated for 20 min with 0.3 ml of test solution and 0.2 ml of 300 μg/ml DNP-As as antigen. The reaction mixture was then centrifuged at 500 *g* for 10 min at 4°C.

The supernatant was assayed for released histamine and the precipitate for the residual cellular histamine according to the method of Shore, Burkhalter & Cohn (1959).

Percentage release of histamine was calculated as (A/A + B) × 100, where A = μg of released histamine, and B = μg of residual cellular histamine.

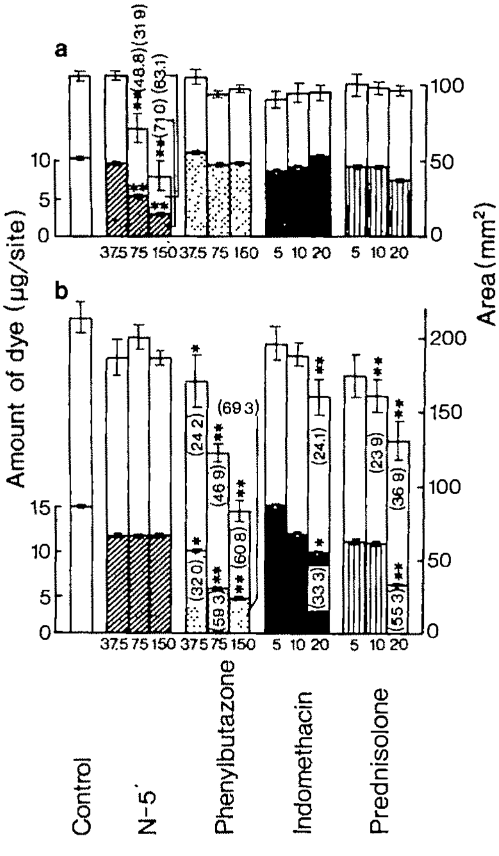


**Figure 2** Effect of drugs on (a) HTA-induced passive cutaneous anaphylaxis (PCA) and (b) anti-BSARS-induced PCA in rats. Each drug was administered orally 30 min before challenge with antigen (DNP-As in (a) and BSA in (b)), see text. Doses of drugs (mg/kg) are given at the base of the columns. Numbers in parentheses indicate inhibition as a percentage of the control value. Mean values from 5 (a) or 6 (b) observations are given. Vertical lines indicate s.e. mean. * $P<0.05$, ** $P<0.01$. The amount of dye leaked (lower parts of columns) as a result of PCA-induced by HTA or by anti-BSARS in controls was 10.4±0.90 and 15.0±0.90 µg/site respectively; the area was 105.1±2.90 and 211.2±10.30 mm², respectively.

The inhibition of histamine release by test drugs was calculated as (1−(D−S/C−S))×100, where C, D, S = percentage release of histamine in controls, in the presence of test drugs, and in the absence of antigen (spontaneous), respectively. Drugs to be tested were dissolved in 1% aqueous solution of $NaHCO_3$.

### *Drugs*

The following drugs were used: phenylbutazone, indomethacin, prednisolone, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate, diphenhydramine hydrochloride, cyproheptadine hydrochloride, carrageenin, Evans blue (Merck), heparin (Novo), bovine serum albumin (Sigma) and Freund's complete adjuvant (Difco).

## **Results**

### *Passive cutaneous anaphylaxis induced with homocytotropic antibodies*

To examine the effect of N-5' at doses of 100 and 200 mg/kg orally on PCA induced by HTA, N-5' was administered at 30, 60, 120 or 240 min before challenge with antigen (DNP-As). Inhibition was most potent at 30 or 60 min pretreatment with N-5', and negligible at 240 min pretreatment (Figure 1). Thirty minutes pretreatment with drugs was therefore used when N-5' was compared with phenylbutazone, indomethacin and prednisolone. The amount of dye leaked as a result of PCA in controls was 10.4±0.90 µg/site and the area 105.1±2.90 mm². N-5' produced a dose-dependent, potent inhibition of the leakage, whereas phenylbutazone, indomethacin or prednisolone had little or no effect (Figure 2a). The PCA was significantly inhibited by the combined pretreatment with 50 mg/kg of diphenhydramine and 10 mg/kg of cyproheptadine (not shown in Figure 2a).

### *Passive cutaneous anaphylaxis induced with anti-bovine serum albumin rabbit serum*

The mean amount of dye leaked as a result of PCA induced by anti-BSARS in controls was 15.0±0.90 µg/site and the area 211.2±10.30 mm² (Figure 2b). Doses of N-5' which potently inhibited homologous PCA mediated by HTA, inhibited only slightly PCA induced by anti-BSARS. On the other hand, heterologous PCA was clearly inhibited by phenylbutazone, indomethacin and prednisolone in a dose-dependent manner. Diphenhydramine (50 mg/kg) and cyproheptadine (10 mg/kg), singly or combined, produced only a slight inhibition (not shown in Figure 2b).

### *Increased vascular permeability caused by histamine or 5-hydroxytryptamine in rats*

N-5', phenylbutazone, indomethacin or prednisolone inhibited only slightly at the doses used the increased vascular permeability caused by histamine and 5-hydroxytryptamine (Figure 3). Diphenhydramine (50 mg/kg p.o.) and cyproheptadine (10 mg/kg p.o.), inhibited increased vascular permeability by 76% and 83%, respectively.

### *Oedema of rat hind paw*

The rat paw oedema induced by carrageenin was inhibited by all tested doses of N-5', phenylbutazone,

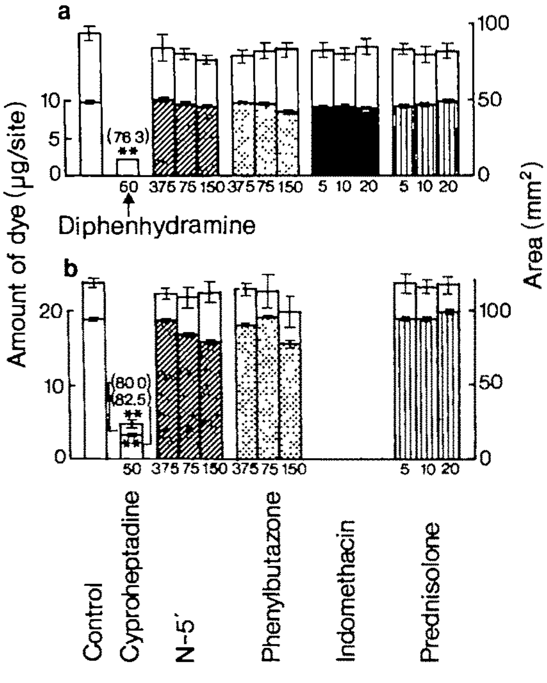


**Figure 3** Influence of drugs on the increased vascular permeability caused by (a) histamine or (b) 5-hydroxytryptamine. Each drug was administered orally 30 min before injection of histamine (10 μg per site in 0.1 ml) or 5-hydroxytryptamine (0.1 μg per site in 0.1 ml). Doses of drugs (mg/kg), percentage inhibition, mean ± s.e. mean (5 observations) and $P$ values are given as in Figure 2. The amount of dye leaked as a result of intradermal injection of histamine and 5-hydroxytryptamine in controls was 9.7 ± 0.43 and 18.9 ± 1.25 μg/site, and the area 94.1 ± 4.63 and 118.9 ± 2.68 mm², respectively. The influence of indomethacin on the increased vascular permeability caused by 5-hydroxytryptamine was not tested.

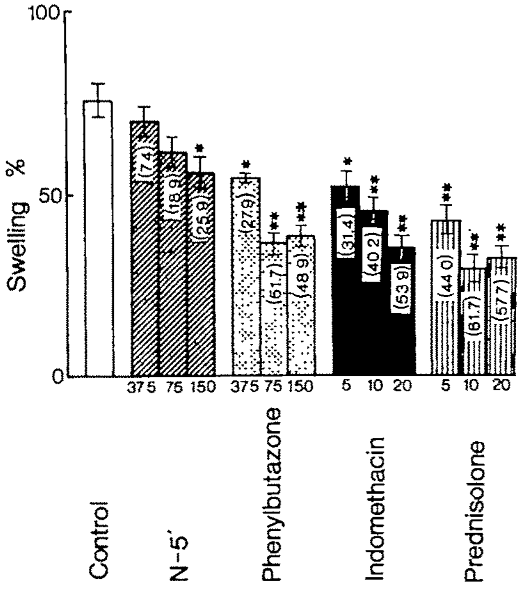


**Figure 4** Influence of drugs on oedema of rat hind paw induced by carrageenin. Each drug was administered orally 30 min before injection of carrageenin (1.0%, 0.1 ml/paw s.c.). Doses of drugs, percentage inhibition, mean ± s.e. mean (5 observations) and $P$ values are given as in Figure 2. Swelling was measured 3 h after carrageenin injection (see text).

indomethacin and prednisolone (Figure 4). N-5′ produced only about 26% inhibition at 150 mg/kg orally, while phenylbutazone, indomethacin and prednisolone were more potent. Inhibition was determined 1, 2, 3, 4 and 5 h after carrageenin injection but the values at 3 h were used, because both the second phase of carrageenin oedema and the inhibitory action of the drugs were most pronounced at that time.

### *Release of histamine from rat peritoneal cells induced by homocytotropic antibodies*

Cells sensitized with HTA were incubated with test drugs 1, 5, 10 or 20 min before challenge with antigen (DNP-As). Inhibition of the allergic histamine release by N-5′ was most potent after 1 min and gradually decreased with longer preincubation times (not shown in Figure). One minute pretreatment with drugs was therefore used (Figure 5).

Figure 5 shows the effect of 1, 10, 100 and 1000 μM N-5′, phenylbutazone, indomethacin and prednisolone on the allergic histamine release from peritoneal cells. Mean total amount of histamine released as a result of antigen-antibody reaction from about $1.5 \times 10^5$ of rat peritoneal mast cells was 2.77 ± 0.130 μg, corresponding to 38.1 ± 1.56% of the total histamine content (7.3 μg/$1.5 \times 10^5$ cells). The spontaneous release without antigen was 11.1 ± 1.87%.

The histamine release was significantly inhibited by 100 and 1000 μM N-5′ but was hardly affected by the drug at a concentration of 10 μM or 1 μM. Phenylbutazone and indomethacin inhibited histamine release by 50% at a concentration of 1000 μM but had no effect at 100, 10 or 1 μM. Prednisolone had no effect at any concentration used.

## Discussion

Homocytotropic antibodies (HTA) resembling human reaginic antibodies in their physicochemical and

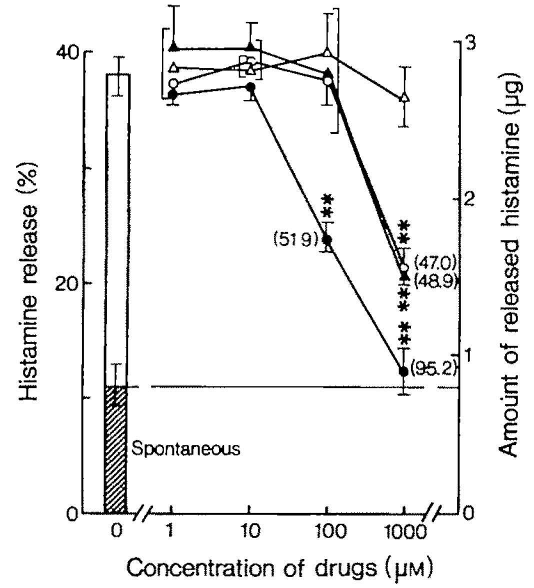


**Figure 5** Influence of N-5′ (●), phenylbutazone (○), indomethacin (△) and prednisolone (▲) on histamine release from rat peritoneal cells induced by homocytotropic antibodies (HTA) and antigen. Each drug was applied 1 min before challenge with antigen (DNP-As) (see text). Each experiment included 6 observations. Vertical lines indicate s.e. mean. ** $P<0.01$. The antigen-antibody reaction released from about $1.5 \times 10^6$ rat peritoneal mast cells 2.77 ± 0.130 μg of histamine or 38.1 ± 1.56% of the total histamine. Spontaneous release of histamine from sensitized cells without antigen was 11.1 ± 1.87%. Numbers in parentheses indicate percentage inhibition (see text).

immunological properties (Mota, 1964; Bianaghi, Benacerraf, Block & Kourilsky, 1964) are induced in rats when antigen is injected with killed *B. pertussis* organisms as adjuvant. The rat antisera used in the present experiments was inactivated by a temperature of 56°C and persisted in the skin for at least 1 week. This supports the view that the homologous PCA involved reagin type antibodies only, and no attempts were made to obtain reagin fractions from rat sera. It is well established that reagin type antibodies induce histamine release by sensitizing mast cells.

N-5′ significantly inhibited the HTA-induced PCA in rats in a dose-dependent manner, while inhibition of anti-BSARS-induced PCA was only slight. Phenylbutazone, indomethacin and prednisolone produced the reverse pattern. Combined treatment with diphenhydramine and cyproheptadine significantly inhibited HTA-induced PCA but not anti-BSARS-induced PCA. This suggests that histamine and 5-hydroxytryptamine may mediate HTA-induced PCA but not anti-BSARS-induced PCA. Our data are supported by the findings of Goose & Blair (1969).

N-5′ and the anti-inflammatory agents inhibited only slightly the increased vascular permeability caused by histamine or 5-hydroxytryptamine. This suggested that the inhibitory action of N-5′ on HTA-induced PCA was not due to antagonism to histamine and 5-hydroxytryptamine but to inhibition of the release of these mediators. The experiments with rat peritoneal cells suggested that N-5′ acts mainly on histamine release. Phenylbutazone and indomethacin inhibited this release only in 1 mM concentrations. Prednisolone was inactive. According to Mota & Ishii (1960), phenylbutazone (1540 μg/ml, about 5 mM) reduced anaphylactic histamine release only by 45%. The report that cortisone did not decrease the anaphylactic histamine release (Trethewie, 1958) agrees with our data. The inhibition by 1 mM phenylbutazone and indomethacin may depend on uncoupling of oxidative phosphorylation and/or membrane stabilization since Miller & Smith (1966), Yamasaki & Saeki (1967) and Ignarro (1971) have shown that phenylbutazone and indomethacin inhibit the mast cell degranulation induced by compound 48/80, by depressing the processes generating ATP and their anti-inflammatory effects are associated with stabilization of the lysosomal membrane.

Rat paw oedema induced by carrageenin was effectively inhibited by the anti-inflammatory agents, but only slightly by N-5′. Yamasaki & Saeki (1967), reported that phenylbutazone, indomethacin, flufenamic acid and mefenamic acid inhibited the inflammatory oedema produced in rats by rabbit anti-rat serum. This oedema was not inhibited by N-5′ (Koda *et al.*, 1976). The pharmacological properties of the anthranilic acid derivative, N-5′ appear to differ from those of anti-inflammatory agents. As proposed by Koda *et al.* (1976), N-5′ could be an anti-allergic clinical drug, effective by oral application. Other pharmacological properties of N-5′ are now under investigation.

The authors are grateful to Dr Y. Sato of The National Institute of Hygienic Science, 1st. Dept. of Bacteriology, Japan, for the generous gift of *B. pertussis*; to Prof. A. Koda, Depts of Pharmacology and Immunology, Gifu College of Pharmacy for pertinent advice; and to M. Ohara for assistance with the manuscript.

## References

BIANAGHI, R.A., BENACERRAF, B., BLOCK, K.J. & KOURILSKY, F.M. (1964). Properties of rat anaphylactic antibody. *J. Immunol.*, **92**, 927–933.

GOOSE, J. & BLAIR, A.M.J.N. (1969). Passive cutaneous anaphylaxis in rat, induced with two homologous reagin-like antibodies and its specific inhibition with DSCG.

*Immunol.*, **16**, 749–760.

IGNARRO, L.J. (1971). Effects of anti-inflammatory drugs on the stability of rat liver lysosomes *in vitro*. *Biochem. Pharmac.*, **20**, 2847–2860.

KODA, A., NAGAI, H., WATANABE, S., YANAGIHARA, Y. & SAKAMOTO, K. (1976). Inhibition of hypersensitivity reactions by a new drug, N-(3′,4′-dimethoxycinnamoyl) anthranilic acid (N-5′). *J. Aller. clin. Immunol.*, **57**, 396–407.

MILLER, W.S. & SMITH, J.G. JR. (1966). Effect of acetylsalicylic acid on lysosomes. *Proc. Soc. exp. Biol. Med.*, **122**, 634–636.

MOTA, I. & ISHII, T. (1960). Inhibition of mast cell disruption and histamine release in rat anaphylaxis *in vitro*. Comparison with compound 48/80. *Br. J. Pharmac. Chemother.*, **15**, 82–87.

MOTA, I. (1964). The mechanism of anaphylaxis. I. Production and biological properties of 'mast cell sensitizing' antibody. *Immunol.*, **7**, 681–699.

SHORE, P.A., BURKHALTER, A. & COHN, V.H. JR. (1959). A method for the fluorometric assay of histamine in tissues. *J. Pharmac. exp. Ther.*, **127**, 182–186.

TADA, T. & OKUMURA, K. (1971). Regulation of homocytotropic antibody formation in the rat. I. Feed-Back Regulation by passively administered antibody. *J. Immunol.*, **106**, 1002–1011.

TRETHEWIE, E.R. (1958). Cortisone and anaphylaxis. *Austral. J. exp. Biol.*, **36**, 275–283.

YAMASAKI, H. & SAEKI, K. (1967). Inhibition of mast-cell degranulation by anti-inflammatory agents. *Archs. int. Pharmacodyn.*, **168**, 166–179.

*(Received January 5, 1976.*
*Revised March 30, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 489–495

# A COMPARISON BETWEEN THE HYPOTHERMIA INDUCED BY INTRAVENTRICULAR INJECTIONS OF THYROTROPIN RELEASING HORMONE, NORADRENALINE OR CALCIUM IONS IN UNANAESTHETIZED CATS

G. METCALF[1] & R.D. MYERS
Laboratory of Neuropsychology, Department of Psychological Sciences, Purdue University,
West Lafayette, Indiana 47907, U.S.A.

1 The hypothermia produced by intraventricular injections of thyrotropin releasing hormone (TRH) in unanaesthetized cats has been investigated.

2 TRH is more potent than either noradrenaline or calcium ions. It is estimated that the equi-potent molar ratio for TRH : noradrenaline : calcium is 1 : 900 : 27,000.

3 TRH injections also produce profuse salivation, tachypnoea, cutaneous vasodilatation and frequently defaecation and vomiting. It is considered that the increased respiration is a major cause of the hypothermia.

4 Prior administration of phentolamine antagonized noradrenaline-induced hypothermia but did not affect hypothermia produced by TRH or calcium ions. Pretreatment with $\alpha$-methyltyrosine did not affect the hypothermia induced by TRH, calcium ions or noradrenaline.

5 The calcium antagonists verapamil and xylocaine did not antagonize hypothermia induced by an injection of calcium ions.

6 The constituent amino acids of TRH did not produce hypothermia either individually or collectively. Thyroxine sodium produced a rise in temperature that was slow in onset, consistent with its known metabolic effects. TSH produced a small hypothermia unrelated to dose.

## Introduction

Thyrotropin releasing hormone (TRH) was isolated from porcine hypothalamus in 1966 (Schally, Bowers, Redding & Barrett). The structure of the compound was later determined to be L-pyroglutamyl-L-histidyl-L-proline amide by Bøler, Enzmann, Folkers, Bowers & Schally (1969) and the molecule was synthesized for the first time in the same year (Folkers, Enzmann, Bøler, Bowers & Schally, 1969). It has been confirmed that the synthetic molecule possesses the same structure and biological properties as the naturally occurring compound. (Bøler *et al.,* 1969; Burgus, Donne, Desiderio, Ward, Vale, Guillemon, Felix, Gillessen & Studer, 1970).

Since its synthesis TRH has been investigated in a variety of neuropharmacological tests. The results obtained indicate that the molecule may have biological properties independent of its role in thyroid metabolism (Plotnikoff, Prange, Breese, Anderson & Wilson, 1972; Prange, Breese, Cott, Martin, Cooper, Wilson & Plotnikoff, 1974; Huidobro-Toro, Scotti de Carolis & Longo, 1974; Horst & Spirt, 1974; Keller, Bartholini & Pletscher, 1974). In addition there is current speculation amongst psychiatrists as to whether TRH is an effective antidepressant (Kastin, Ehrensing, Schalch & Anderson, 1972; Prange, Wilson, Lara, Alltop & Breese, 1972; Mountjoy, Price, Weller, Hunter, Hall & Dewar, 1974; Benkert, Gordon & Martschke, 1974) or that it may aid in the differential diagnosis of depressive illness (Ehrensing, Kastin, Schalch, Friesen, Vargas & Schally, 1974).

In 1974 Metcalf reported that intraventricular injections of TRH produced hypothermia in unanaesthetized cats but hyperthermia in unanaesthetized rabbits and noted that noradrenaline had previously been reported to produce similar temperature effects to TRH in both of these species (Feldberg & Myers, 1963, 1964; Cooper, Cranston & Honour, 1965). Intrahypothalamic administration of calcium ions has also been reported to produce hypothermia in cats (Myers & Veale, 1971). The present investigations, in which the hypothermia produced by intraventricular injection of TRH in the cat was compared to the hypothermia produced by noradrenaline or calcium administered by a similar route, were undertaken to ascertain whether TRH acted indirectly via either one of these other two agents and, in addition, to determine whether other thyroid hormones had similar effects.

---

[1] Present address: Pharmaceutical Division, Reckitt & Colman, Dansom Lane, Kingston-upon-Hull, HU8 7DS.

A brief account of some aspects of this work has been published previously (Metcalf & Myers, 1975b).

## Methods

Twenty-one cats of either sex were used. At the time of implantation all the animals were in the weight range 2.0–6.0 kg.

### *Surgical procedures*

The procedures previously described (Metcalf, Myers & Redgrave, 1975) were used to implant each animal with two guide tubes. Each guide tube consisted of a 28 mm length of 18 gauge stainless steel tubing (Popper Company, New York, NY) fitted with an indwelling stylet of the same length and tip bevel. For each animal the guide tubes were positioned so that their tips rested 4 mm above the lateral ventricle on either side of the brain (i.e. 3 mm lateral and 7 mm caudal to Bregma). During surgery the correct positioning of each guide tube was verified by the rapid inflow of artificial CSF into the ventricle under gravity using a 32 mm (20 ga) injector. After they had been lowered to the appropriate position the guide tubes were anchored to the calvarium with cranioplast cement and stainless steel screws. A screw top polyethylene pedestal was fastened over the guide tubes to protect them and prevent subsequent infection.

### *Experimental procedure*

All experiments were performed at a normal laboratory temperature of 20–22°C. Before each experiment began, a thermistor probe (type 401, Yellow Springs Instrument Company, Ohio) was inserted 10 cm into the animal's colon and held in place by surgical tape wrapped gently around the base of the tail. The rectal temperature was recorded continuously on a multi-channel potentiometric recorder. A base-line period of at least 1 h was recorded before an injection was made. Periodically throughout the experiment respiratory rate was counted and ear temperature assessed by touch. Animals were unrestrained throughout the experiments. Although animals were used repeatedly over a number of weeks at least 7 days were allowed to elapse between successive experiments.

Intraventricular injections were made with a 1 ml sterile, disposable syringe connected by means of a length of polyethylene tubing (PE90) to a 32 mm injector constructed of 20 gauge stainless steel tubing. To give an injection the stylet was removed from the guide tube and the injector cannula was lowered so that its tip rested in the lateral ventricle 4 mm beyond the guide tube. The polyethylene tubing was then disconnected from the syringe and a small amount of solution allowed to flow into the ventricle under the influence of gravity to ensure that contact with the ventricular lumen had been established. Then the syringe was reconnected to the tubing and a 200 μl injection was made over a period of 2 minutes. After the injection the stylet was replaced immediately and the effects of the drug injection assessed over a period of at least 4 hours.

### *Injection solutions*

All solutions were prepared with glass distilled, deionized water. An artificial CSF solution (Myers, 1971) containing the chloride salts of $Na^+$ 145.0 mM, $Ca^{2+}$ 1.3 mM, $K^+$ 3.5 mM and $Mg^{2+}$ 1.0 mM was used both for control injections and as the vehicle for drug injections. The drugs used were TRH (Reckitt & Colman), (−)-noradrenaline bitartrate, α-methyltyrosine methyl ester, DL-thyroxine sodium, thyrotropin (Sigma), phentolamine mesylate (Ciba), xylocaine (Astra), verapamil hydrochloride (Knoll, AG). Doses were calculated in terms of the active ionic species. Each solution was sterilized by passing it through a sterile, disposable 0.22 μm Swinnex Millipore filter just before injection. The injection cannulae and polyethylene tubing were stored in 0.0013% benzalkonium chloride solution and the whole injection system was flushed repeatedly with the solution to be injected just before use.

### *Site verification*

At the conclusion of the experiments, 200 μl indian ink was injected into the ventricle concerned. The animal was then killed by an overdose of pentobarbitone sodium and the brain fixed by successive retrograde perfusions of isotonic saline and 10% buffered neutral formalin through the thoracic aorta after the heart had been clamped off. The ventricular system was then exposed (Myers, 1971) and the distribution of the injection volume ascertained by inspection.

## Results

### *Comparison of the hypothermia produced by intraventricular injections of TRH, noradrenaline or calcium ions*

Doses of 0.1–10.0 μg TRH given intravenously had no effect on body temperature. In contrast intraventricular injections of 50–200 ng TRH produced a

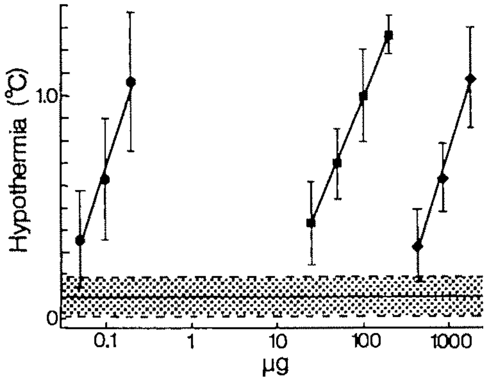


Figure 1 Dose-related hypothermias induced by the intraventricular injection of either thyrotropin releasing hormone (TRH, ●), noradrenaline (■) or calcium (◆). Each point is the mean from 4–6 experiments. Vertical lines show s.e. mean. Hypothermia was measured as the minimum temperature obtained within 2 h of injection. The shaded area represents the effect (mean ± s.e. mean) produced by control injections of vehicle.

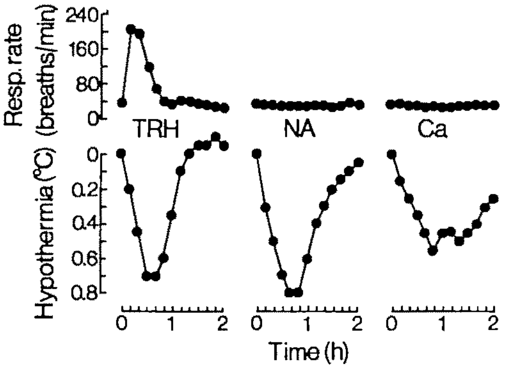


Figure 2 Changes in body temperature (°C) and respiratory rate (breaths/min) produced by intraventricular injections of 0.1 μg thyrotropin releasing hormone (TRH), 50 μg noradrenaline or 40 mM excess calcium into individual unanaesthetized cats. All injections made at time 0.

dose-related hypothermia (Figure 1). This was immediate in onset, developed rapidly and reached its maximum value in approximately 30–45 minutes. In addition animals treated with TRH invariably exhibited profuse salivation, tachypnoea or even open-mouthed panting and cutaneous vasodilatation as evidenced by increased ear temperatures. Frequently the animals defaecated or vomited. All of these latter effects were usually completed within the first 30 min after injection. Thereafter body temperature returned to normal whilst the animal lay quietly and appeared tired or sedated.

The administration of either noradrenaline (25–200 μg) or calcium (20–80 mM in excess of that normally found in CSF, for comparison these doses have been expressed as μg in Figure 1) by the intraventricular route, also produced hypothermia in cats. In both cases the effect produced was related to dose (Figure 1) but in neither case were these compounds comparable in potency to TRH. Approximate equi-effective dose-ratios TRH : noradrenaline : calcium were calculated to be 1 : 500 : 9000. Taking molecular weight into account the equi-effective molar ratio TRH : noradrenaline : calcium may be recalculated as 1 : 900 : 27,000 thus making TRH the most potent hypothermic agent yet reported.

Animals treated with noradrenaline or calcium did not exhibit the wide variety of effects observed after the intraventricular administration of TRH. After either noradrenaline or calcium vasodilatation was evident and maximum hypothermia was attained in 30–60 min but neither the profuse salivation nor the rapid respiration observed after TRH were seen. Rarely did animals treated with noradrenaline or calcium defaecate or vomit. In almost every case they appeared quiet or sedated.

### *The correlation between increased respiratory rate and hypothermia*

Figure 2 compares the time courses for hypothermias and respiratory changes observed after 100 ng TRH, 50 μg noradrenaline and 40 mM excess calcium. These doses were selected for subsequent comparisons between TRH, noradrenaline and calcium. Examination of Figure 2 shows that whilst the effects of these doses on body temperature were comparable, the effects on respiratory rate were not. Only TRH produced intense tachypnoea after i.c.v. injection and this tachypnoea actually preceded the onset of hypothermia.

To ascertain whether these two effects were related, a scattergram was constructed in which the fall in temperature produced by various doses of TRH (25–200 ng i.c.v) was plotted against the maximum respiratory rate observed after the same injection. Figure 3 illustrates the plotted data and the regression line which was fitted to them ($y = 53 + 82x$). The positive correlation of 0.82 between fall in temperature and increased respiratory rate was significant ($t = 3.97$; $P < 0.001$).

### *The effect of phentolamine pretreatment on the hypothermia induced by noradrenaline, calcium or TRH*

The minimum effective dose of phentolamine for these experiments was found to be 125 μg. Doses in excess

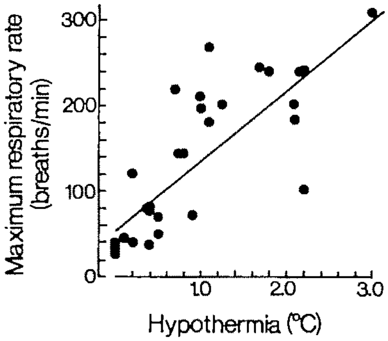


**Figure 3** Relationship between respiratory rate (breaths/min) and hypothermia (°C) induced in unanaesthetized cats by intraventricular injection of various doses of thyrotropin releasing hormone (TRH).

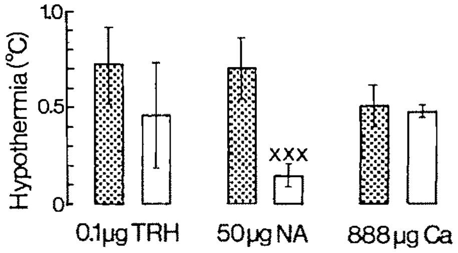


**Figure 4** The effect of phentolamine (125 µg, i.c.v.) pretreatment on the hypothermia induced by intraventricular injections of 0.1 µg thyrotropin releasing hormone (TRH), 50 µg noradrenaline (NA) or 40 mM excess calcium in unanaesthetized cats. Shaded columns represent controls and open columns represent the post-phentolamine values. Values are mean obtained from 4 experiments. Vertical lines show s.e. mean. xxx indicates the result is significantly different from the control, $P<0.001$.

of 250 µg themselves caused a fall in temperature of 0.5–0.75° which lasted 2 or 3 hours, whilst doses less than 125 µg had only marginal effects on the noradrenaline response. The results obtained when 50 µg noradrenaline, 40 mM excess calcium or 100 ng TRH were administered 15 min after 125 µg phentolamine are illustrated in Figure 4. It can be seen that prior treatment with phentolamine significantly decreased the hypothermia produced by subsequent intraventricular injection of noradrenaline ($t=4.62$; $P<0.001$). In contrast pretreatment with phentolamine produced no effect on the hypothermia which resulted from the intraventricular injection of a solution containing either excess calcium ions ($t=0.12$; $P>0.1$) or TRH ($t=0.86$; $P>0.1$).

### *The effect of α-methyltyrosine pretreatment*

To determine whether intact cerebral noradrenaline stores are necessary for TRH or calcium ions to exert their hypothermic actions, the hypothermic effects of TRH, calcium and noradrenaline were compared both before and after depletion of central noradrenaline stores with α-methyltyrosine. Depletion of noradrenaline was obtained with a total dose of 100 mg α-methyltyrosine administered in divided doses by the intraventricular route. This dosage regimen has previously been shown to deplete cerebral noradrenaline in the cat (Cranston, Hellon, Luff & Rawlins, 1972). The results obtained are illustrated in Figure 5. It can be seen that the degree of hypothermia induced by an intraventricular injection of 50 µg noradrenaline, administered approximately 12 h after the last dose of α-methyltyrosine, is not significantly less than that obtained under control conditions ($t=0.46$; $P>0.1$). Similarly the ability of both TRH ($t=0.90$; $P>0.1$) and calcium ($t=0.85$; $P>0.1$) to provoke hypothermia was unaffected by depletion of central noradrenaline stores.

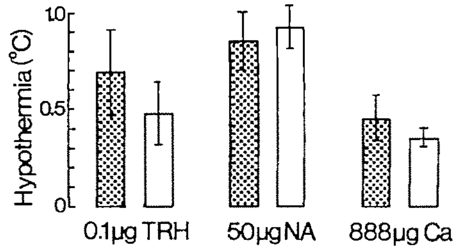


**Figure 5** The effect of α-methyltyrosine (100 mg, i.c.v. in divided doses) pretreatment on the hypothermia induced by intraventricular injections of 0.1 µg thyrotropin releasing hormone (TRH), 50 µg noradrenaline (NA) or 40 mM excess calcium in unanaesthetized cats. Values are mean obtained from 4 experiments. Vertical lines show s.e. mean. Shaded columns represent controls and open columns the post-α-methyltyrosine values.

### *Experiments with calcium antagonists*

The calcium antagonists verapamil (25–250 µg) and xylocaine (100–400 µg) (see Phillis, 1974) were used in an attempt to inhibit selectively calcium-induced hypothermia. Neither substance inhibited this effect of calcium.

### *The effect of intraventricular injections of L-histidine, L-pyroglutamic acid or L-prolineamide on body temperature*

To determine whether the hypothermia observed after intraventricular injection of TRH could be attributed to a particular fragment of the complete molecule, the amino acids composing TRH were tested for their individual effects on body temperature. The results set out in Table 1 indicate that none of the three amino acids concerned has any effect on cats' body temperature; neither did a mixture of all three acids.

### *The effect of intraventricular injections of thyrotropin or thyroxine on body temperature*

TRH is known to stimulate the direct release of thyrotropin (TSH) from the adenohypophysis and this in turn releases thyroxine (T4) from the thyroid gland. The effect of these compounds on body temperature was determined to ascertain whether TRH acted indirectly to produce hypothermia. Thyroxine (0.25–1.0 µg, i.c.v.) produced a dose-related rise in temperature (Table 2). There was a latent period of approximately 2 h before the rise began, after which temperature rose slowly but steadily to reach a maximum value about 4 h after the injection. Body temperature then remained elevated for several hours. During the hyperthermia the animals appeared subdued or sedated but exhibited none of the other effects observed after TRH administration. Cats treated with TSH (0.2–0.8 i.u., i.c.v.) exhibited small falls in body temperature (Table 2). The hypothermia was not related to the dose administered, was gradual in onset (peak hypothermia achieved in 1–2 h) and long in duration (2–4 hours). Immediately after the injection animals frequently mewed continuously for several minutes and appeared restless. Thereafter they appeared relaxed and playful.

## Discussion

TRH has been shown to be a more potent hypothermic agent than either noradrenaline or calcium when injected into the cerebral ventricles of unanaesthetized cats. The equi-potent molar ratio for TRH : noradrenaline : calcium was estimated to be 1 : 900 : 27,000. As well as this great quantitative difference between TRH and the other two hypothermic agents there were marked qualitative differences between the effects observed after injection. After either noradrenaline or calcium the animals exhibited only cutaneous vasodilatation and appeared sedated. By comparison animals dosed with TRH exhibited signs of autonomic stimulation: profuse salivation, vomiting or diarrhoea together with marked increases in respiratory rate. Statistical analysis determined a positive correlation between the increased respiratory rate and the fall in temperature produced by TRH, so that at least one mechanism contributing towards the hypothermia is suggested.

The different behavioural effects observed after TRH suggests that the peptide acts by a different mechanism from either noradrenaline or calcium. This tentative conclusion is supported by results from a parallel study in which microinjection of TRH into the hypothalamus failed to produce hypothermia or any of the behavioural effects seen after i.c.v. injection (Myers, Metcalf & Rice, unpublished observation). Previous workers have established that intrahypothalamic administration of noradrenaline (Feldberg & Myers, 1965) or calcium (Myers & Veale, 1971) will both reliably produce hypothermia in cats.

As a result of their experiments with a variety of $\alpha$- and $\beta$-adrenoceptor stimulants, Rudy & Wolf (1971) concluded that the central receptor with which noradrenaline reacted to induce hypothermia was $\alpha$ in nature. Other results with adrenoceptor blocking agents (Feldberg & Saxena, 1971) are in

**Table 1** The effects of intraventricular injections of histidine, pyroglutamic acid and prolineamide on body temperature in the cat

| *Amino acid* | *Dose (ng)* | $\Delta^t_{60}$°C |
|---|---|---|
| Control | — | −0.10 ± 0.09 |
| L-Histidine | 100 | +0.05 ± 0.20 |
| L-Prolineamide | 100 | +0.05 ± 0.06 |
| L-Pyroglutamic | 100 | −0.10 ± 0.12 |
| Equi-molar mixture of all three acids | 30, 30, 30 | +0.08 ± 0.09 |

Values are mean ± s.e. mean obtained from 4 experiments. $\Delta^t_{60}$ is the maximum changed in body temperature produced within 1 h after injection.

**Table 2** The effect of intraventricular injections of DL-thyroxine sodium or thyroid stimulating hormone (TSH) on body temperature in the cat

| | *Dose* | $\Delta^t_{max}$°C |
|---|---|---|
| DL-Thyroxine sodium | 0.25 µg | +0.21 ± 0.08 |
| | 0.5 µg | +0.53 ± 0.17 |
| | 1.0 µg | +0.90 ± 0.19 |
| TSH | 0.2 iu | −0.31 ± 0.05 |
| | 0.4 iu | −0.49 ± 0.13 |
| | 0.8 iu | −0.20 ± 0.02 |

Values are mean ± s.e. mean obtained from 4 experiments. $\Delta^t_{max}$ is the maximum change in body temperature produced within 8 h of the injection. TSH dosage is in International units (iu).

keeping with this hypothesis. In the present experiments phentolamine, an α-receptor antagonist, was used successfully to antagonize hypothermia produced by a subsequent injection of noradrenaline. The same dose of phentolamine had no discernible effect on the hypothermia produced by either calcium ions or TRH. Thus neither TRH nor calcium appear to produce their hypothermic effects by stimulation of central α-receptors. The inference that central noradrenaline mechanisms are not involved in either TRH or calcium hypothermias was confirmed by the experiments with α-methyltyrosine. This latter compound decreases endogenous stores of noradrenaline by inhibiting the biosynthetic pathway for the catecholamine (Levitt, Spector, Sjoerdsma & Udenfriend, 1965) and it has been shown that α-methyltyrosine pretreatment abolishes the hypothermic activity of intraventricular tyramine (Metcalf & Myers, 1975a); a compound known to act indirectly via the release of endogenous noradrenaline from nerve endings (see Smith, 1973). Pretreatment with α-methyltyrosine had no effect on the hypothermia produced by noradrenaline, calcium or TRH implying that none of these three compounds act indirectly by releasing endogenous noradrenaline from nerve endings.

The experiments with calcium antagonists were not conclusive. These compounds have been used by a number of workers to antagonize the effects of calcium ions on excitable tissues, and are thought to act by inhibiting the passage of calcium through membranes (see Phillis, 1974). In our experiments neither of the calcium antagonists used had any effect on the fall in body temperature induced by subsequent injections of calcium. Further experiments in which calcium antagonists are micro-injected into tissue sites known to be sensitive to calcium are indicated.

TRH is a tripeptide composed of pyroglutamic acid and histidine together with the amide of proline. When these fragments of the TRH molecule were examined for hypothermic activity they were found to be ineffective, both individually or collectively, indicating that the complete TRH molecule is necessary to evoke hypothermia. Green & Grahame-Smith (1974) also concluded that the complete molecule was necessary for a behavioural hyperactivity syndrome observed in rats. Physiologically, TRH is known to stimulate the release of TSH from the anterior pituitary gland and this in turn stimulates production of the thyroid hormones, which act peripherally to stimulate metabolism and cause heat production. Both TSH and thyroxine were examined for their effects on body temperature after intraventricular injection. Thyroxine did not produce hypothermia at any of the doses examined, in fact it produced a gradual rise in temperature that was slow in onset and consistent with its known actions as a metabolic stimulant (Andersson, Ekman, Gale & Sundsten, 1963; Beleslin & Samardzić, 1973). Although intraventricular injections of TSH produced slight hypothermia the effect was gradual in onset, more protracted in duration and not related to dose, at least in the dose range investigated. In addition, none of the other symptoms observed after TRH was seen after TSH. Thus the two effects are considered to be unrelated.

To summarise: the hypothermia observed after intraventricular injection of TRH in unanaesthetized cats is a function of the complete molecule, appears to be independent of the compound's endocrine activity and unrelated to the hypothermia produced by similar injections of noradrenaline or calcium ions. The primary cause of the hypothermia may be the loss of heat caused by stimulation of the respiratory rate.

The authors are grateful to Mr J.C. Rice, for his technical assistance during these experiments. The research was supported in part by grant GB 35380 from the National Science Foundation and U.S. Office of Naval Research contract N-0014-67-A-0226-0003. Dr D.J. Shaefer of the Pharmaceutical Division, Reckitt and Colman, Hull, England supplied the TRH and Professors Kleinsorge and Oberdorf of Knoll, A.G., Germany kindly supplied the verapamil. G.M. is grateful to the Wellcome Trustees for their award of a Travel Grant.

## References

ANDERSSON, G., EKMAN, L., GALE, C.C. & SUNDSTEN, J.W. (1963). Control of thyrotrophic hormone (TSH) secretion by the "Heat Loss Center". *Acta physiol. scand.*, **59**, 12–33.

BELESLIN, D.B. & SAMARDZIĆ, R. (1973). Effect of thyroxine on the body temperature after its intraventricular injection into conscious cats. *J. Physiol. Lond.*, **238**, 27–28P.

BENKERT, O., GORDON, A. & MARTSCHKE, D. (1974). The comparison of thyrotropin releasing hormone, luteinising hormone-releasing hormone and placebo in depressive patients using a double-blind cross-over technique. *Psychopharmacologia (Berl.)*, **40**, 191–198.

BØLER, J., ENZMANN, F., FOLKERS, K., BOWERS, C.Y. & SCHALLY, A.V. (1969). The identity of chemical and hormonal properties of the thyrotrophin releasing hormone and pyroglutamyl-histidyl-proline amide. *Biochem. biophys. Res. Commun.*, **37**, 705–710.

BURGUS, R., DONNE, Th.F., DESIDERIO, D.M., WARD, D.M., VALE, W., GUILLEMON, R., FELIX, D.M., GILLESSEN, D. & STUDER, R.O. (1970). Biological activity of synthetic polypeptide derivatives related to the structure of hypothalamic TRF. *Endocrinology*, **86**, 573–582.

COOPER, K.E., CRANSTON, W.I. & HONOUR, A.J. (1965). Effects of intraventricular and intrahypothalamic injection of noradrenaline and 5HT on body temperature in conscious rabbits. *J. Physiol., Lond.*, **181**, 852–864.

CRANSTON, W.I., HELLON, R.F., LUFF, R.H. & RAWLINS, M.D. (1972). Hypothalamic endogenous noradrenaline and thermoregulation in the cat and rabbit. *J. Physiol. Lond.*, **223**, 59–67.

EHRENSING, R.H., KASTIN, A.J., SCHALCH, D.S., FRIESEN, H.G., VARGAS, J.R. & SCHALLY, A.B. (1974). Affective state and thyrotropin and prolactin responses after repeated injections of thyrotropin-releasing hormone in depressed patients. *Am. J. Psychiatry*, **131**, 714–718.

FELDBERG, W. & MYERS, R.D. (1963). A new concept of temperature regulation by amines in the hypothalamus. *Nature, Lond.*, **200**, 1325.

FELDBERG, W. & MYERS, R.D. (1964). Effects on temperature of amines injected into the cerebral ventricles. A new concept of temperature regulation. *J. Physiol. Lond.*, **173**, 226–236.

FELDBERG, W. & MYERS, R.D. (1965). Changes in temperature produced by microinjections of amines into the anterior hypothalamus of the cat. *J. Physiol. Lond.*, **177**, 239–245.

FELDBERG, W. & SAXENA, P.N. (1971). Effects of adrenoceptor blocking agents on body temperature. *Br. J. Pharmac.*, **43**, 543–554.

FOLKERS, K., ENZMANN, F., BØLER, J., BOWERS, C.Y. & SCHALLY, A.V. (1969). Discovery of modification of the synthetic tripeptide sequence of the thyrotropin releasing hormone having activity. *Biochem. biophys. Res. Commun.*, **37**, 123–126.

GREEN, A.R. & GRAHAME-SMITH, D.G. (1974). TRH potentiates behavioural changes following increased brain 5-hydroxytryptamine accumulation in rats. *Nature, Lond.*, **251**, 524–526.

HORST, W.D. & SPIRT, N. (1974). A possible mechanism for the antidepressant activity of thyrotropin hormone. *Life Sciences*, **15**, 1073–1082.

HUIDOBRO-TORO, J.P., SCOTTI DE CAROLIS, A. & LONGO, V.G. (1974). Action to two hypothalamic factors (TRH, MIF) and of Angiotensin II on the behavioural effects of L-dopa and 5-hydroxytryptophan in mice. *Pharmacology, Biochemistry & Behaviour*, **2**, 105–109.

KASTIN, A.J., EHRENSING, R.H., SCHALCH, D.S. & ANDERSON, M.S. (1972). Improvement in mental depression with decreased thyrotropin response after administration of thyrotropin-releasing hormone. *Lancet*, **ii**, 740–742.

KELLER, H.H., BARTHOLINI, G. & PLETSCHER, A. (1974). Enhancement of cerebral noradrenaline turnover by thyrotropin-releasing hormone. *Nature, Lond.*, **248**, 528–529.

LEVITT, M., SPECTOR, S., SJOERDSMA, A. & UDENFRIEND, S. (1965). Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharmac. exp. Ther.*, **148**, 1–8.

METCALF, G. (1974). TRH: a possible mediator of thermoregulation. *Nature, Lond.*, **252**, 310–311.

METCALF, G. & MYERS, R.D. (1975a). Further evidence concerning the role of brain noradrenaline in mammalian thermoregulation. *J. Pharm. Pharmac.*, **27**, 616–617.

METCALF, G. & MYERS, R.D. (1975b). Investigations concerning TRH-induced hypothermia in cats. *Br. J. Pharmac.*, **55**, 273P–274P.

METCALF, G., MYERS, R.D. & REDGRAVE, P.C. (1975). Temperature and behavioural responses induced in the unanaesthetized cat by the central administration of RX 72601, a new anticholinesterase. *Br. J. Pharmac.*, **55**, 9–15.

MOUNTJOY, C.W., PRICE, J.S., WELLER, M., HUNTER, P., HALL, R. & DEWAR, J.H. (1974). A double-blind crossover sequential trial of oral thyrotropin-releasing hormone in depression. *Lancet*, **i**, 958–960.

MYERS, R.D. (1971). General laboratory procedures. In *Methods in Psychobiology*, ed. Myers, R.D. Vol. I. pp. 27–65. London: Academic Press.

MYERS, R.D. & VEALE, W.L. (1971). The role of sodium and calcium ions in the hypothalamus in the control of body temperature of the unanaesthetized cat. *J. Physiol. Lond.*, **212**, 411–430.

PHILLIS, J.W. (1974). Minireview: The role of calcium in the central effects of biogenic amines. *Life Sciences*, **14**, 1189–1201.

PLOTNIKOFF, N.P., PRANGE, A.J., BREESE, G.R., ANDERSON, M.S. & WILSON, I.C. (1972). Thyrotropin releasing hormone: Enhancement of dopa activity by a hypothalamic hormone. *Science*, **178**, 417–418.

PRANGE, A.J., BREESE, G.R., COTT, J.M., MARTIN, B.R., COOPER, B.R., WILSON, I.C. & PLOTNIKOFF, N.P. (1974). Thyrotropin releasing hormone: antagonism of pentobarbital in rodents. *Life Sciences*, **14**, 447–455.

PRANGE, A.J., WILSON, I.C., LARA, P.P., ALLTOP, L.B. & BREESE, G.R. (1972). Effects of thyrotropin-releasing hormone in depression. *Lancet*, **ii**, 999–1002.

RUDY, T.A. & WOLF, H.H. (1971). The effect of intrahypothalamically injected sympathomimetic amines on temperature regulation in the cat. *J. Pharmac. exp. Ther.*, **179**, 218–235.

SCHALLY, A.V., BOWERS, C.Y., REDDING, T.W. & BARRETT, J.F. (1966). Isolation of thyrotropin-releasing-factor (TRF) from porcine hypothalamus. *Biochem. biophys. Res. Commun.*, **25**, 265–269.

SMITH, A.D. (1973). Mechanisms involved in the release of noradrenaline from sympathetic nerves. *Br. med. Bull.*, **29**, 123–129.

*(Received January 27, 1976.*
*Revised May 20, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 497–504

# EXTRA-VESICULAR BINDING OF NORADRENALINE AND GUANETHIDINE IN THE ADRENERGIC NEURONES OF THE RAT HEART: A PROPOSED SITE OF ACTION OF ADRENERGIC NEURONE BLOCKING AGENTS

A. GIACHETTI & R.A. HOLLENBECK[1]
Department of Pharmacology, University of Texas Southwestern Medical School, Dallas, Texas, U.S.A.

1 The binding and efflux characteristics of [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline were studied in heart slices from rats which were pretreated with reserpine and nialamide.

2 Binding of both compounds occurred at extra-vesicular sites within the adrenergic neurone. After a brief period of rapid washout, the efflux of [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline proceeded at a steady rate. The efflux of both compounds appeared to occur from a single intraneuronal compartment.

3 (+)-Amphetamine accelerated the efflux of [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline; this effect was inhibited by desipramine.

4 Unlabelled guanethidine and amantadine also increased the efflux of labelled compounds. Cocaine in high concentrations increased slightly the efflux of [$^{14}$C]-guanethidine but not that of [$^{3}$H]-noradrenaline.

5 Heart slices labelled with [$^{3}$H]-noradrenaline became refractory to successive exposures to releasing agents although an appreciable amount of labelled compound was still present in the slices.

6 It is suggested that [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline are bound at a common extra-vesicular site within the adrenergic neurone. Binding of guanethidine to the extra-vesicular site may be relevant to its pharmacological action, i.e., the blockade of adrenergic transmission.

## Introduction

In an attempt to elucidate the mechanism of action of adrenergic neurone blockers Abbs and colleagues (Abbs & Robertson, 1970; Abbs & Pycock, 1973; Abbs & Dodd, 1974) studied the intracellular distribution of noradrenaline in the spleen and in heart following the administration of bretylium and certain guanidinium compounds to cats and rats. These studies revealed significant changes in the intraneuronal distribution of transmitter, which were temporally related to the onset of adrenergic neurone blockade; however, it is difficult to assign a functional role to subcellular fractions of tissue homogenates since they may not correspond to functional sites existing in intact tissues.

An insight into the intraneuronal locus of action of adrenergic neurone blockers may be obtained by examining the interactions of these agents with amphetamine, a drug which readily prevents and reverses the blockade of adrenergic transmission (Laurence & Rosenheim, 1960; Day & Rand, 1963).

Since amphetamine displaces noradrenaline mainly from an extra-vesicular compartment (Farnebo, 1971) it is conceivable that the ability to prevent or reverse the adrenergic blockade and the noradrenaline releasing properties are causally related and effected at the same site.

Other observations suggest that adrenergic neurone-blocking agents may induce a blockade of impulse transmission through an action on an extra-vesicular site within the neurone. For example, Shand, Morgan & Oates (1973), by examining the nerve-induced release of guanethidine and bethanidine from adrenergic nerve endings, indicated that the portion of guanidinium compounds retained in storage vesicles does not contribute to the development of adrenergic blockade and they postulated an extra-vesicular site of action for adrenergic neurone-blocking drugs.

In view of the evidence implicating a possible interaction of adrenergic neurone blockers with extra-vesicular stores of noradrenaline in the production of adrenergic blockade, we have examined the characteristics of guanethidine and noradrenaline binding at extra-vesicular sites within the adrenergic neurone and factors which influence that binding.

---

[1] Present address: Lab. of Preclinical Pharmacology, N.I.M.H., St. Elizabeth's Hospital, Washington, D.C. 20032, U.S.A.

## Methods

### *Preparation and incubation of rat heart slices*

Female Sprague-Dawley rats (150–200 g) were used. Reserpine (2 mg/kg) and nialamide (100 mg/kg), when given, were administered subcutaneously 18 and 3 h, respectively, before killing. Heart ventricle slices (0.3 mm, 50–80 mg) were obtained with a Harvard tissue slicer. The slices were incubated in Krebs-Ringer bicarbonate (pH 7.4) supplemented with tricarboxylic acid intermediates (Dengler, Spiegel & Titus, 1961) as well as ascorbic acid (50 mg/litre). After equilibration (15 min) slices were incubated with labelled noradrenaline ((±)-[$^3$H]-noradrenaline) or guanethidine ([$^{14}$C]-guanethidine). Due to the difference in specific activity between these compounds (see below), and in order to obtain tissue levels of isotope adequate to perform efflux studies, heart slices were incubated with 0.1 μM [$^3$H]-noradrenaline for 30 min or with 1 μM [$^{14}$C]-guanethidine for 60 minutes. All incubations and washouts were performed at 37° in the presence of 95% $O_2$ and 5% $CO_2$.

### *Efflux of labelled compounds*

After incubation, the efflux of labelled compounds from the heart slices was followed by placing the tissue in 5 ml of the buffer and transferring to fresh buffer at 5 min intervals for periods of up to 70 minutes.

The ability of drugs to release labelled compounds from the slices was studied by including the test drug in the washout medium for brief periods of time. Thus, slices labelled with [$^3$H]-noradrenaline were exposed to drugs for a single 5 min interval after 55 min of washout, unless otherwise indicated. In experiments involving the release of [$^{14}$C]-guanethidine, slices were exposed to drugs after 30 min of efflux and the exposure time was prolonged to 3 periods of 5 min each. This longer exposure was necessary in order to obtain levels of radioactive compound in the medium significantly higher than the baseline efflux. It should be mentioned that baseline efflux was subtracted from each sample (see calculations).

The effect of a membrane transport inhibitor on release of labelled compounds was investigated by adding desipramine (10 μM) to the washout medium for 10 min before and during the interval of exposure to the releasing agent.

### *Analytical determinations*

Radioactivity in the washout medium was determined by the addition of 2 ml of medium to 10 ml scintillation fluid (Aquasol, New England Nuclear) and counting in a liquid scintillation spectrometer (Beckman) to a statistical accuracy of ±3%. The efficiency of counting averaged 39% for $^3$H and 64% for $^{14}$C.

Tissue levels of labelled noradrenaline were determined by homogenization of the tissue in 29 parts of 0.4 N $HClO_4$ followed by centrifugation. Aliquots (0.5 ml) of the supernatant were placed in 10 ml scintillation solution and counted as above. In preliminary experiments incubation medium and perchloric acid extracts were absorbed onto alumina and eluted as described by Anton & Sayre (1962). The amount of radioactivity in the eluate after correction for recovery (53.4%) was equal to the radioactivity present before purification, thus indicating that total radioactivity represented unchanged [$^3$H]-noradrenaline. Results are therefore based on total radioactivity. Oxidative deamination can be excluded *a priori* in the present experiments since a monoamine-oxidase inhibitor was always employed.

[$^{14}$C]-guanethidine sulphate was purified before use by extraction into butanol-heptane mixture as described by Medina, Giachetti & Shore (1969) for debrisoquine and purity was confirmed by ascending paper chromatography.

In experiments involving labelled guanethidine, radioactivity was determined directly in the incubation medium; tissue levels were determined by the same procedure described for [$^3$H]-noradrenaline.

### *Calculations*

The uptake of labelled compounds is expressed in terms of the ratio of radioactivity in the tissue (ct $\text{min}^{-1}\ \text{g}^{-1}$) corrected for cell water (85%) to that in the incubation medium (ct $\text{min}^{-1}\ \text{ml}^{-1}$). Rate of efflux of labelled compounds during washout is expressed as percentage efflux with time and was calculated as follows:

$$\%\ \text{efflux} = \frac{\dfrac{\text{ct/min released during 5 min}}{\text{sample interval}}}{\begin{array}{c}\text{ct/min present in tissue at}\\ \text{beginning of sample interval}\end{array}} \times 100$$

To determine the level of labelled compound remaining in the tissues at any given 5 min sampling interval during washout, the total ct/min determined for each 5 min sample was added successively to the final tissue ct/min level, beginning with the last washout sample and ending with the first. Percentage efflux is a rate function and represents that portion of labelled compound released during a 5 min washout interval relative to the total radioactivity present in the tissue slice at the beginning of that interval.

In the case of tissue slices which were subjected to the amine-releasing activity of drugs, percentage release is expressed as the difference in percentage efflux between the 5 min drug exposure interval and the preceding 5 min washout interval. The latter value represents a point at which the washout of labelled agent proceeds at a constant rate (baseline).

### *Drugs*

Compounds used in the investigation included (+)-amphetamine sulphate, amantadine hydrochloride, guanethidine sulphate, cocaine hydrochloride, desipramine hydrochloride (DMI), nialamide base, reserpine phosphate, potassium chloride; (±)-[7-$^{3}$H]-noradrenaline, 13 Ci/mM (New England Nuclear) and [$^{14}$C]-guanethidine sulphate, 4.1 mCi/mM (Ciba-Geigy).

## Results

### *Accumulation of [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline by rat ventricular slices*

Incubation of heart slices with [$^{14}$C]-guanethidine or [$^{3}$H]-noradrenaline resulted in the rapid accumulation of both compounds within the slices as indicated by the ratios of tissue concentration relative to the medium concentration of compounds (Table 1).

Reserpine pretreatment decreased very considerably the accumulation of noradrenaline even when tissue monoamine-oxidase was inhibited by nialamide. In contrast, the initial uptake of [$^{14}$C]-guanethidine was not affected although the overall accumulation noted at longer incubation times was reduced (Table 1).

To ensure that the labelled compounds were accumulated within the adrenergic neurone when vesicular storage was impaired by reserpine, cocaine (10 μM), a potent inhibitor of the amine pump, was included in the incubation medium. In these conditions the accumulation of the [$^{3}$H]-noradrenaline was inhibited by 90%, while that of [$^{14}$C]-guanethidine was inhibited by 51%; this different sensitivity to the action of cocaine suggests that part of the [$^{14}$C]-guanethidine accumulates in non-neuronal cells.

These results indicate that in the absence of vesicular storage function, the adrenergic neurone maintains the ability to accumulate these compounds in the axoplasm where binding occurs at extra-vesicular sites.

The following series of experiments illustrates the latter point.

### *Efflux of [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline*

Heart slices taken from reserpine-treated rats were allowed to accumulate [$^{14}$C]-guanethidine or [$^{3}$H]-noradrenaline and then transferred at regular intervals of time to tracer-free medium. The characteristics of the efflux of [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline from heart slices are depicted in Figure 1a. In this graph the radioactivity appearing in the medium is expressed as percentage of efflux against time (see Methods section), i.e. the graph represents the fractional rate of efflux of isotopic compounds from the slices. Analysis of Figure 1 reveals an initial rapid rate of efflux of each compound lasting 10–15 min followed by a very slow and steady rate which amounts to 7–10% efflux per 5 minutes. This latter phase suggests that efflux of compounds is mainly from a single intracellular compartment.

Another indication that [$^{14}$C]-guanethidine exists in the slice in two kinetically different compartments is seen in the lower portion of Figure 1 where the isotope remaining in the tissue at the end of 5 min intervals, expressed as percentage of total taken up, is plotted against time. The same desaturation pattern is observed for [$^{3}$H]-noradrenaline (Figure 1, lower

**Table 1** Accumulation of [$^{14}$C]-guanethidine ([$^{14}$C]-G) and [$^{3}$H]-noradrenaline ([$^{3}$H]-NA) by rat heart slices: effect of reserpine treatment

| | *Tissue to medium ratios ± s.e. mean* | | | |
|---|---|---|---|---|
| *Incubation time (min)* | *Control* | | *Reserpine* | |
| | *[$^{14}$C]-G* | *[$^{3}$H]-NA* | *[$^{14}$C]-G* | *[$^{3}$H]-NA* |
| 10 | 1.15±0.093 | 1.91±0.091 | 1.20±0.041 | 0.66±0.027 |
| 30 | 2.21±0.082 | 2.17±0.037 | 1.89±0.122 | 1.17±0.084 |
| 60 | 3.53±0.367 | 5.19±0.420 | 2.68±0.163 | 1.58±0.099 |

Each result is the mean of 3–5 experimental values. Reserpine-treated rats received 2 mg/kg subcutaneously, and nialamide (100 mg/kg s.c.), 18 h and 3 h respectively before they were killed. Control designates rats receiving nialamide (100 mg/kg s.c.) 3 h before they were killed. Slices were incubated with 0.1 μM [$^{3}$H]-noradrenaline or with 1 μM [$^{14}$C]-guanethidine as described in the Methods section.

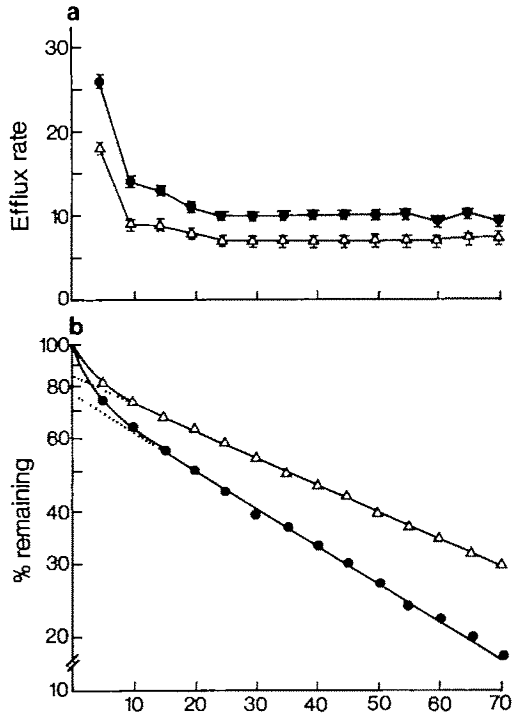


**Figure 1** Rate of efflux (a) of [$^3$H]-noradrenaline (●) and [$^{14}$C]-guanethidine (△) during washout, and (b) semilogarithmic plot of labelled compounds remaining in rat heart slices during washout expressed as percentage of total isotope taken up. Rate is expressed as percentage of isotope present which leaves the tissues during 5 min period (see Methods section). Dotted lines in plot (b) are extrapolations to t=0 to determine half-times of desaturation. Heart slices in this and following experiments were taken, unless otherwise indicated, from rats treated with reserpine (2 mg/kg s.c. 18 h) and nialamide (100 mg/kg s.c. 3 h). Each point is the mean of 6–8 experiments. Vertical lines show s.e. mean.

panel). Extrapolation of the slow component of each curve allows the estimation of the half-time for efflux of [$^3$H]-noradrenaline (33 min) and [$^{14}$C]-guanethidine (48 minutes). In the early phase of washout, the efflux is too rapid to permit an accurate determination of half-time of efflux.

### *Release of [$^{14}$C]-guanethidine and [$^3$H]-noradrenaline by amphetamine*

To examine the ability of amphetamine to induce the release of accumulated [$^{14}$C]-guanethidine and [$^3$H]-noradrenaline from reserpine-treated rat tissue, heart slices were exposed to amphetamine for 15 or 5 min, respectively, at a point during washout when efflux of either labelled compound had reached a steady rate. The release expressed as percentage efflux of the labelled compounds above baseline efflux is shown in Figure 2. The extent of release induced by amphetamine is similar for [$^{14}$C]-guanethidine and [$^3$H]-noradrenaline and, in the range of concentrations examined, appears to be related to the molarity of amphetamine.

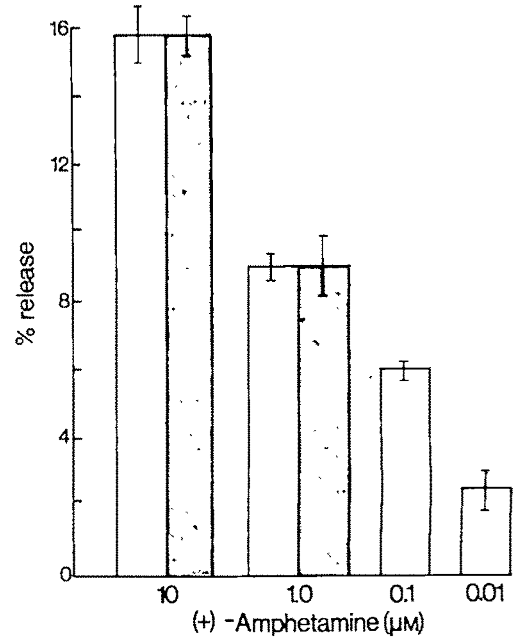


**Figure 2** Effect of amphetamine on efflux of [$^3$H]-noradrenaline (open columns) or [$^{14}$C]-guanethidine (solid columns) from heart slices. Release expresses the portion of efflux of labelled compound above baseline washout when concentrations of (+)-amphetamine were present for 5 min ([$^3$H]-noradrenaline) or 15 min ([$^{14}$C]-guanethidine). Each column is the mean of 6–8 observations. Vertical lines show s.e. mean.

The overall amount of amphetamine-releasable noradrenaline was quite small (accounting for about 6% of the noradrenaline taken up by the tissue) and the pool apparently was fully depleted during the interval of amphetamine exposure. In fact, a second exposure to amphetamine (10 μM) 25 min after the first exposure failed to promote release of the labelled compound although the slices still contained appreciable amounts of radioactivity. In other experiments we investigated the ability of amphetamine to release [$^{14}$C]-guanethidine and [$^3$H]-noradrenaline accumulated in heart slices taken from untreated rats. The results shown in Table 2 indicating enhancement of the releasing properties of amphetamine in reserpine-treated slices relative to

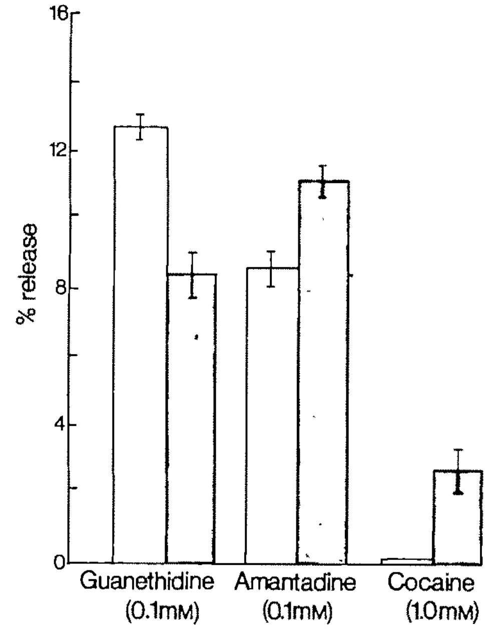


**Figure 3** Effect of drugs on efflux of [$^3$H]-noradrenaline (open columns) and [$^{14}$C]-guanethidine (solid columns). Release expressed as in Figure 2. Drugs were present in washout medium for 5 min ([$^3$H]-noradrenaline) or 15 min ([$^{14}$C]-guanethidine). On the abscissa scale guanethidine (0.1 mM) represents unlabelled compound. Each column is the mean of 6 observations. Vertical lines show s.e. mean.

**Table 2** Release of labelled guanethidine or noradrenaline by amphetamine: effect of reserpine pre-treatment

| | *% Release above baseline efflux* | |
|---|---|---|
| *Treatment* | *[$^{14}$C]-G* | *[$^3$H]-NA* |
| Control | 4.6 ± 0.17 | 2.75 ± 0.12 |
| Reserpine* | 15.7 ± 0.59 | 15.7 ± 0.87 |

Each value is the mean of 4 to 6 experiments ± s.e. mean. Slices were labelled and subsequently exposed to amphetamine (10 μM) as described in the Methods section.

* Reserpine (2 mg/kg s.c.) 18 h before rats were killed. Control and reserpine-treated rats received nialamide (100 mg/kg s.c.) 3 h before killing.

normal slices suggest that reserpine treatment unmasks an amphetamine-releasable pool of either compound.

### *Release of [$^{14}$C]-guanethidine and [$^3$H]-noradrenaline by other agents*

The ability of unlabelled guanethidine and of the anti-parkinson agent amantadine to release [$^{14}$C]-guanethidine and [$^3$H]-noradrenaline accumulation in heart slices taken from rats pre-treated with reserpine and nialamide (see Methods section) was also examined. The results summarized in Figure 3 show that both agents promote release of the labelled compounds although they are much weaker than amphetamine.

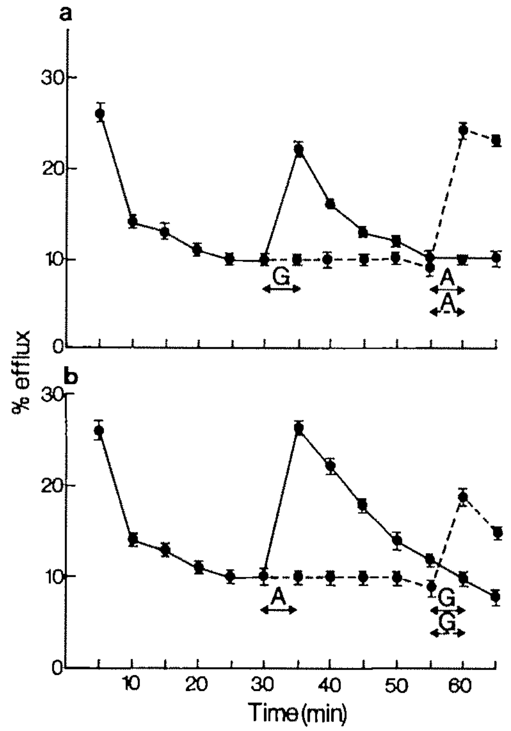


**Figure 4** Efflux of [$^3$H]-noradrenaline from rat heart slices: (a) effect of exposure to unlabelled guanethidine (G 0.1 mM) and later to (+)-amphetamine (A 10 μM). Drugs were present at times indicated by solid arrow. In (b) exposure to (+)-amphetamine and later to unlabelled guanethidine. Dashed lines and arrows represent efflux in heart slices exposed to a single releasing agent at 55 min during washout. Percent efflux expressed as in Figure 1 (mean of 4 observations for both (a) and (b); vertical lines show s.e. mean).

Since guanethidine and amantadine are known to inhibit the membrane amine transport mechanism (Mitchell & Oates, 1970; Farnebo, Fuxe, Goldstein, Hamberger & Ungerstedt, 1971) it was of interest to determine whether the increase in efflux of labelled compounds could be due to blockade of their reuptake through the neuronal membrane. However, cocaine, a potent amine uptake inhibitor, when tested at 0.1 mM proved to be ineffective as a releaser of [$^3$H]-noradrenaline and promoted release of [$^{14}$C]-guanethidine only to a minor extent (Figure 3).

To determine whether amphetamine and

guanethidine, when used as releasing agents, were acting upon the same pool of accumulated labelled compound, a series of experiments was performed in which tissue slices loaded with [$^{3}$H]-noradrenaline were exposed to either amphetamine (10 μM) or guanethidine (0.1 mM) followed by a later exposure to the agents in reverse order. Figure 4 shows that both agents promote labelled amine efflux upon initial exposure, while a later challenge with the alternate agent fails to produce additional release. Thus, guanethidine (0.1 mM) increased the efflux of [$^{3}$H]-noradrenaline (12% above baseline efflux) whereas exposure to amphetamine (10 μM) in the same preparation after 55 min of wash promoted no additional release of isotope (Figure 4a). The same pattern of release was observed when exposure of the tissues to the releasing agents was reversed (Figure 4b). Failure to promote efflux of [$^{3}$H]-noradrenaline by a second exposure to a releasing agent was apparently not due to disappearance of labelled compound from the slice, since analysis of these tissues revealed a level of isotope comparable to slices which had been challenged by a single exposure.

### *Effect of membrane transport inhibition on amphetamine-induced release of [$^{14}$C]-guanethidine and [$^{3}$H)-noradrenaline*

When heart tissue slices loaded with [$^{14}$C]-guanethidine or [$^{3}$H]-noradrenaline were exposed to desipramine (10 μM) for 10 min before and during exposure to amphetamine (10 μM), during washout, inhibition of release was observed (Table 3). Since desipramine was incapable of promoting release in these conditions, it appears that inhibition of release might be due to inability of amphetamine to gain access to the releasable store of labelled compounds.

**Table 3** Release of [$^{14}$C]-guanethidine ([$^{14}$C]-G) and [$^{3}$H]-noradrenaline ([$^{3}$H]-NA) by (+)-amphetamine from rat heart slices: effect of desipramine

| *Drug (conc 10 μM)* | *% Release above baseline efflux* | |
|---|---|---|
| | *[$^{14}$C]-G* | *[$^{3}$H]-NA* |
| Amphetamine | 15.7 ± 0.59 | 15.7 ± 0.87 |
| Desipramine + amphetamine | 11.1 ± 0.62 | 8.5 ± 0.32 |
| Desipramine | 0 | 0 |

Results are the mean ± s.e. mean of 5 observations. Heart slices incubated with desipramine for 10 min before challenge with (+)-amphetamine during washout of labelled compounds (see Methods section). Rats treated with reserpine and nialamide as in legend of Table 1.

## Discussion

Adrenergic neuronal blocking agents inhibit sympathetic neurotransmission by preventing the release of noradrenaline in response to nerve stimulation (Boura & Green, 1957; Abercrombie & Davies, 1963). It has been suggested that the adrenergic neuronal blockers displace noradrenaline from an intraneuronal pool critical for the liberation of transmitter by nerve impulses (Abbs & Robertson, 1971; Shand *et al.*, 1973; Abbs & Dodd, 1974).

To examine the possibility of an extragranular location as a site for interaction between guanethidine and noradrenaline, the function of the amine storage granules in the adrenergic neurone was inhibited by reserpine administration. Table 1 shows that such treatment reduces the ability of the heart slice to accumulate [$^{3}$H]-noradrenaline and, to a lesser extent, [$^{14}$C]-guanethidine; similar findings for the accumulation of guanethidine have been reported previously (Brodie, Chang & Costa, 1965).

Evidence that part of the accumulated [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline may be bound to a single subcellular site was found in the efflux experiments. A large fraction (about 55%) of the total accumulated labelled compound left the tissues upon washout at a steady rate following the rapid disappearance of unspecifically bound amine. Of that labelled compound remaining in the tissues following the rapid phase of efflux, approximately 7% of the [$^{14}$C]-guanethidine and 10% of the [$^{3}$H]-noradrenaline left the tissues every 5 min possibly indicating dissociation from a binding site followed by efflux.

Since amphetamine has been shown to be capable of releasing extragranularly bound noradrenaline (Lundborg & Waldeck, 1971; Farnebo, 1971) as well as reversing adrenergic nerve blockade (Laurence & Rosenheim, 1960; Follenfant & Robson, 1970), the agent was selected as a pharmacological tool to aid in relating noradrenaline binding and release characteristics to those of the adrenergic neuronal blocker, guanethidine. Amphetamine (10 μM) was found capable of releasing about 16% of the total [$^{14}$C]-guanethidine or [$^{3}$H]-noradrenaline remaining in the tissue after washout of unspecifically bound amine (Figure 2).

The size of the amphetamine-releasable store of [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline in the adrenergic neurone is apparently increased by reserpine treatment (Table 2). This enhancement of an amphetamine-releasable amine store has been observed for noradrenaline by other investigators (Farnebo, 1971; Enna & Shore, 1974) and probably reflects a different intraneuronal distribution of labelled compounds accumulated when the granular storage function is inhibited by reserpine.

Further evidence for a common or related intraneuronal binding site for guanethidine and noradrenaline is found in the experiments testing the ability of unlabelled guanethidine and amantadine (an anti-parkinson agent possessing some sympathomimetic properties) to promote release of accumulated [$^{14}$C]-guanethidine and [$^{3}$H]-noradrenaline from heart slices (Figure 3). Amantadine, which can reverse adrenergic neuronal blockade (unpublished observations), also displayed an ability to promote efflux of the labelled compounds, thus strengthening the relevance of the extra-vesicular compartment for the maintenance of the adrenergic neurone blockade. The finding that the [$^{3}$H]-noradrenaline bound extra-vesicularly is readily exhausted by a single exposure to amphetamine is indicative of a small and discrete compartment which cannot be refilled by the relatively large pool of isotope contained within the neurone. It is also apparent that unlabelled guanethidine releases [$^{3}$H]-noradrenaline from the same compartment since its releasing action can be abolished by previous challenge with amphetamine (Figure 4). Failure to elicit further release of [$^{3}$H]-noradrenaline by a second exposure to amphetamine or guanethidine might be due to binding of the releasing agent to extra-vesicular sites. The results of this study indicate that guanethidine interacts with extra-vesicular sites in a manner similar to that of [$^{3}$H]-noradrenaline and indeed it might replace the transmitter at these sites. That a similar mechanism might be operant for amphetamine can be inferred by the similarities with the releasing action of guanethidine. It is of interest to note that while desipramine or cocaine do not affect the accumulation of amphetamine in rat tissues (Ross & Renyi, 1964; Thoenen, Huerlimann & Haefely, 1968) desipramine reduces considerably the releasing effect of amphetamine on compounds accumulated extra-vesicularly (Table 3). These results confirm and extend the work of Lundborg & Waldeck (1971) who suggested that extra-vesicular noradrenaline is selectively localized in a site accessible via the membrane amine transport mechanism.

It is tempting to speculate that reversal of guanethidine-induced adrenergic neuronal blockade by sympathomimetic amines may be due to displacement of the blocker from an intraneuronal binding site. Reversal of adrenergic neuronal blockade has been shown to be accompanied by a loss of blocker from adrenergically innervated tissue (Kirpekar & Furchgott, 1972; Shand *et al.*, 1975). Although inhibitors of the neuronal membrane transport mechanism (e.g. cocaine) have been found to reverse adrenergic neuronal blockade, they appear to do so more gradually than amphetamine (Gerkins, McCulloch & Wilson, 1969) and are apparently more effective in preventing blockade than reversing it (Day, 1962) and do not reverse blockade persistently (Kirpekar, Wakade, Dixon & Prat, 1969). These observations suggest that while amphetamine may owe part of its adrenergic neuronal blockade reversal properties to its ability to prevent reuptake of adrenergic neuronal blockers, a major mechanism might be the displacement of the neurone blocker from intraneuronal binding sites. The selective displacement of adrenergic neuronal blocking drugs from a critical intraneuronal site might then allow such a store to refill with noradrenaline and thus restore nerve function. Some workers have concluded that adrenergic neurone blockers inhibit transmission by depressing the excitability of adrenergic nerve terminals (Haeusler, Haefely & Huerlimann, 1969). This interpretation does not account for the immediate and complete reversal of adrenergic blockade observed after administration of amphetamine and related compounds. Binding of adrenergic neurone blocking agents to a selective intraneuronal site might explain the rapid onset of nerve blockade as well as the rapid reversal seen after amphetamine. Displacement by amphetamine of bound [$^{14}$C]-guanethidine noted in this investigation has also been recently reported to occur for [$^{3}$H]-bretylium accumulated in rat atria (Ross & Gosztonyi, 1975). The role of extra-vesicularly bound noradrenaline in adrenergic transmission is speculative at the present. Evidence that the depolarization of neuronal membrane by nerve impulses promotes the release of noradrenaline from storage vesicles is firmly established (Malmfors, 1969; Farnebo, 1971) but little is known about the intermediate steps involved in this process.

It is possible that membrane depolarization activates a small intraneuronal pool of noradrenaline located near the terminal membrane; the liberation of noradrenaline from this site triggers the release of larger amounts of transmitter from storage vesicles. This concept is analogous to the mechanism postulated for acetylcholine in ganglionic transmission (Koelle, 1962); the adrenergic neurone would then utilize one of its characteristic constituents, i.e. noradrenaline in a sequential fashion to transmit information. Substitution of noradrenaline bound at the extravesicular site by guanethidine would result in suppression of transmitter release from storage vesicles. Day & Rand (1963), after examining the interactions of guanethidine and dexamphetamine concluded that these agents act competitively at 'receptors' located at the 'store' of noradrenaline. Our results support this interpretation and suggest that the 'receptors' alluded to are extra-vesicular binding sites in the adrenergic neurones.

This work was supported by U.S.P.H.S. Grant MH–05831, HL 19300–01.

**References**

ABBS, E.T. & DODD, M.G. (1974). The relation between the adrenergic neurone blocking and noradrenaline depleting actions of some guanidine derivatives. *Br. J. Pharmac.*, **51**, 237–247.

ABBS, E.T. & PYCOCK, C.J. (1973). The effects of bretylium on the subcellular distribution of noradrenaline and on adrenergic nerve function in rat heart. *Br. J. Pharmac.*, **49**, 11–22.

ABBS, E.T. & ROBERTSON, M.I. (1970). Selective depletion of noradrenaline: a proposed mechanism of the adrenergic neurone blocking action of bretylium. *Br. J. Pharmac.*, **38**, 776–791.

ABERCOMBIE, G.F. & DAVIES, B.N. (1963). The action of guanethidine with particular reference to the sympathetic nervous system. *Br. J. Pharmac.*, **20**, 171–177.

ANTON, A.H. & SAYRE, D.F. (1962). A study of the factors affecting the aluminum oxide-trihydroxyindole procedure for the analysis of catecholamines. *J. Pharmac. exp. Ther.*, **138**, 360–375.

BOURA, A.L.A. & GREEN, A.F. (1957). The action of bretylium: adrenergic neurone blocking and other effects. *Br. J. Pharmac. Chemother.*, **12**, 306–311.

BRODIE, B.B., CHANG, C.E. & COSTA, E. (1965). On the mechanism of action of guanethidine and bretylium. *Br. J. Pharmac.*, **25**, 171–178.

DAY, M.D. (1962). Effect of sympathomimetic amines on the blocking action of guanethidine, bretylium and xylocholine. *Br. J. Pharmac.*, **18**, 421–439.

DAY, M.D. & RAND, M.J. (1963). Evidence for a competitive antagonism of guanethidine by dexamphetamine. *Br. J. Pharmac.*, **20**, 17–28.

DENGLER, H.J., SPIEGEL, H.E. & TITUS, E.O. (1961). Effects of drugs on uptake of isotopic norepinephrine by cat tissue. *Nature, Lond.*, **191**, 816–817.

ENNA, S.J. & SHORE, P.A. (1974). On the nature of the adrenergic neuron extragranular amine binding site. *J. Neural Transmission*, **35**, 125–133.

FARNEBO, L. (1971). Effect of d-amphetamine on spontaneous and stimulation-induced release of catecholamines. *Acta physiol. scand., Suppl.*, **371**, 45–52.

FARNEBO, L., FUXE, K., GOLDSTEIN, M., HAMBERGER, B. & UNGERSTEDT, U. (1971). Dopamine and noradrenaline releasing action of amantadine in the central and peripheral nervous system: a possible mode of action in Parkinson's disease. *Eur. J. Pharmac.*, **16**, 27–38.

FOLLENFANT, M.J. & ROBSON, R.D. (1970). The antagonism of adrenergic neuron blockade by amphetamine and dexamphetamine in the rat and guinea-pig. *Br. J. Pharmac.*, **38**, 792–801.

GERKINS, J.F., McCULLOCH, M.W. & WILSON, J. (1969). Mechanism of the antagonism between guanethidine and dexamphetamine. *Br. J. Pharmac.*, **35**, 563–572.

HAEUSLER, G., HAEFELY, W., HUERLIMANN, A. (1969). On the mechanism of the adrenergic nerve blocking action of bretylium. *Naunyn-Schmiedebergs Arch. Pharmak.*, **265**, 260–277.

KIRPEKAR, S.M. & FURCHGOTT, R.F. (1972). Interaction of tyramine and guanethidine in the spleen of the cat. *J. Pharmac. exp. Ther.*, **180**, 38–46.

KIRPEKAR, S.M., WAKADE, A.R., DIXON, W. & PRAT, J.C. (1969). Effect of cocaine, phenoxybenzamine, and calcium on the inhibition of norepinephrine output from the cat spleen by guanethidine. *J. Pharmac. exp. Ther.*, **165**, 166–175.

KOELLE, G.B. (1962). A new general concept of the neurohumoral function of acetylcholine and acetylcholinesterase. *J. Pharm. Pharmac.*, **14**, 65–90.

LAURENCE, D.R. & ROSENHEIM, E. (1960). Clinical effects of drugs which prevent the release of adrenergic transmitter. *Ciba Foundation Symposium on Adrenergic Mechanisms*, ed. Vane, J.R., Wolstenholme, G.E.W. & O'Connor, H. pp. 201–208. London: J. & A. Churchill, Ltd.

LUNDBORG, P. & WALDECK, B. (1971). On the mechanism of amphetamine induced release of reserpine-resistant $^3$H-noradrenaline and $^3$H-alpha-methylnoradrenaline. *Acta pharmac. tox.*, **30**, 339–347.

MALMFORS, T. (1969). Histochemical studies on the effect of nerve impulses on exogenous catecholamines taken up into the adrenergic nerves of reserpine-pretreated animals. *Pharmacology*, **2**, 193–208.

MEDINA, M.A., GIACHETTI, A. & SHORE, P.A. (1969). On the physiological disposition and possible mechanism of the antihypertensive action of debrisoquin. *Biochem. Pharmac.*, **18**, 891–901.

MITCHELL, J.R. & OATES, J.A. (1970). Guanethidine and related agents. I. Mechanism of selective blockade of adrenergic neurons and its antagonism by drugs. *J. Pharmac. exp. Ther.*, **172**, 100–107.

ROSS, S.B. & RENYI, A.L. (1964). Blocking action of sympathomimetic amines on the uptake of tritiated noradrenaline by mouse cerebral cortex tissue *in vitro*. *Acta pharmac. tox.*, **21**, 226–239.

ROSS, S.E. & GOSZTONYI, T. (1975). On the mechanism of the accumulation of $^3$H-bretylium in peripheral sympathetic nerves. *Naunyn-Schmiedebergs Arch. Pharmak.*, **288**, 283–293.

SHAND, D.G., MORGAN, D.H. & OATES, J.A. (1973). The release of guanethidine and bethanidine by splenic nerve stimulation: a quantitative evaluation showing dissociation from adrenergic blockade. *J. Pharmac. exp. Ther.*, **184**, 73–80.

THOENEN, H., HUERLIMANN, A. & HAEFELY, W. (1968). Mechanism of amphetamine accumulation in the isolated perfused heart of the rat. *J. Pharm. Pharmac.*, **20**, 1–11.

*(Received February 14, 1976.*
*Revised May 25, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 505–515

# CARDIOVASCULAR EFFECTS OF DOPAMINE AFTER CENTRAL ADMINISTRATION INTO CONSCIOUS CATS

M.D. DAY[1] & A.G. ROACH[2]
Pharmacological Laboratories, Department of Pharmacy, University of Aston, Birmingham, B4 7ET

1 Dopamine (30 and 45 µg) administered intracerebroventricularly (i.c.v.) to a group of 10 conscious normotensive cats caused dose-related increases in blood pressure and heart rate. In 4 of these animals the initial cardiovascular stimulant effects of i.c.v. dopamine were followed by hypotension and bradycardia.

2 α-Methyldopamine (30 and 45 µg i.c.v.) produced qualitatively similar responses to dopamine except that the cardiovascular stimulant effects were smaller and the secondary depressant effects somewhat more prolonged.

3 Both stimulant and depressant effects of i.c.v. dopamine and α-methyldopamine were greatly inhibited by autonomic ganglion blockade or by adrenergic neurone blockade.

4 The cardiovascular stimulant effects of both i.c.v. dopamine and i.c.v. α-methyldopamine were selectively inhibited by β-adrenoceptor blocking agents whilst the cardiovascular depressant effects of these substances were abolished by the α-adrenoceptor blocker phentolamine or by the dopamine-β-hydroxylase inhibitor disulfiram.

5 Haloperidol by either i.c.v. or the intravenous route abolished both cardiovascular stimulant and depressant effects of i.c.v. dopamine, whilst pimozide by either route inhibited only the cardiovascular stimulant effects.

6 In 2 cats, injection of dopamine into the cisterna magna produced predominantly depressant effects on the cardiovascular system except with a higher dose which induced biphasic responses.

## Introduction

Numerous reports have appeared in recent years concerning the mechanism of the cardiovascular depressant effects usually seen after the central administration of noradrenaline and related sympathomimetic amines in conscious and anaesthetized cats and rats (for instance, Henning, 1973; 1975; Van Zwieten, 1973; Day & Roach, 1974a,b; de Jong, Nijkamp & Bohus, 1975; Struyker-Boudier, Smeets, Brouwer & van Rossum, 1975). In general, it appears that hypotension and bradycardia result from stimulation of α-adrenoceptors in the lower brain stem (see references above) and in the anterior hypothalamus (Toivola & Gale, 1970; Struyker-Boudier, Smeets, Brouwer & van Rossum, 1974). In addition, β-adrenoceptors may also be concerned in the central control of blood pressure since intracerebroventricular (i.c.v.) isoprenaline in conscious cats usually caused cardiovascular stimulant effects (Day & Roach, 1972; 1973; 1974a).

Dopamine is the immediate physiological precursor of noradrenaline, and in addition, itself possesses neurotransmitter functions in the brain. However, little work has been reported concerning a possible role for dopamine in the central regulation of blood pressure. McCubbin, Kaneko & Page (1960) found that i.c.v. dopamine in anaesthetized dogs had no effect on blood pressure unless monoamine oxidase had previously been inhibited when it produced hypotension, bradycardia and marked impairment of the carotid sinus pressor reflex. Intracerebroventricular dopamine has been reported to cause hypotension and bradycardia in rats and cats (Baum & Shropshire, 1973; Heise & Kroneberg, 1973; Finch, Hersom & Hicks, 1975), and also when applied to the ventral surface of the brain stem (Bloch, Bousquet, Feldman, Velly & Schwartz, 1974). Finch *et al.* (1975) found that inhibition of dopamine-β-hydroxylase abolished the cardiovascular depressant effects of α-methyldopamine in conscious rats suggesting that these effects were mediated via formation of α-methylnoradrenaline. This observation casts doubt on the validity of previous reports concerning the central actions of dopamine since part, at least, of any observed effects may have been due to the formation of noradrenaline.

---

[1] Present address: Department of Physiology and Pharmacology, University Hospital and Medical School, Nottingham NG7 2UH.

[2] Present address: Laboratoires d'Etudes et de Recherches Scientifiques, 58 Rue de la Glacière, Paris 75013, France.

We have studied the effects of centrally-administered dopamine on the blood pressures and heart rates of conscious normotensive cats both before and after inhibition of dopamine-β-oxidase.

## Methods

Ten cats of either sex, weighing between 2.5–4.5 kg were used in this study. Under halothane anaesthesia arterial and venous cannulae were inserted by the method of Day & Whiting (1972). The blood pressure recording system consisting of catheters, valve and transducer were tested to ensure that the frequency response of the system was adequate to give reliable measurements of both systolic and diastolic pressures. The system was tested with an electromagnetic pressure generator (Ardill, Fentem & Wellard, 1967). The recording system used in the cat experiments gave no damping of sinusoidal pulses with frequencies up to 5 Hz when compared with a similar transducer connected directly to the pulse generator. In the present experiments resting heart rates were of the order of 100–130 beats/min and rarely exceeded 200 beats/min in response to drugs and thus the system was considered adequate for the measurement of both systolic and diastolic pressures. A modified Collison Cannula (Cooling, Day & Roach, 1974) was implanted in the left lateral cerebral ventricle (i.c.v.) as described by Feldberg & Sherwood (1953). At least seven days elapsed following operation before each cat was used in an experimental study.

### *Injections into the cisterna magna*

Two cats previously cannulated for i.c.v. injection were additionally cannulated for injections into the cisterna magna (i.c.m.). A cannula guide was made from a No. 0 hypodermic needle based upon the guide described by Feldberg, Myers & Veale (1970). The needle shaft was cut and filed to a length of 18 mm. The guide was cemented to the skull so that its tip rested just above the atlanto-occipital membrane. A No. 1 gauge hypodermic needle was inserted into the guide until it punctured the membrane; cerebrospinal fluid rose up the needle. The injection needle was fixed in this position with quick drying epoxyresin and capped with a sealed piece of polyethylene tubing.

### *Drugs*

Drugs infused i.c.v. or i.c.m. were dissolved in sterile 0.9% w/v NaCl solution (saline) ('Steriflex', Allen & Hanbury's Ltd) except where otherwise stated. Intracerebroventricular and i.c.m. drug administrations were made in volumes of 100 and 50 µl respectively and were infused over a 4 min period with a constant infusion pump (Scientific and Research Instruments Ltd).

The drugs used were: dopamine hydrochloride (Sigma), (–) α-methyldopamine hydrochloride (Merck, Sharp & Dohme), isoprenaline sulphate (BDH), pempidine tartrate (May & Baker), hexamethonium bromide (Koch-Light Labs.), tetramethylammonium chloride (BDH), bethanidine sulphate (Burroughs Wellcome), guanethidine monosulphate (Ciba), (+) and (±)-propranolol (ICI), (+) and (±)-alprenolol (Astra), lignocaine hydrochloride (BDH), procaine hydrochloride (BDH), phentolamine methane sulphonate (Ciba), haloperidol and pimozide (Janssen Pharmaceuticals) and disulfiram (tetraethylthiuram disulphide, BDH).

The doses in the text of (+)-alprenolol, haloperidol and pimozide are quoted as base and all other doses as the salts mentioned above. Disulfiram was injected intraperitoneally as a suspension in sterile saline using compound powder of Tragacanth BPC as the suspending agent. Haloperidol was dissolved in dilute lactic acid and the pH of the solution adjusted to 4.8 with 0.1 N NaOH. Pimozide was dissolved in 0.1% w/v tartaric acid and the pH adjusted to 5.5 with 0.1N NaOH.

**Table 1** Maximal increases in blood pressure and heart rate (mean ± s.e.) induced by two dose levels of each of dopamine and α-methyldopamine administered into the lateral cerebral ventricles of a group of conscious cats which responded to both drugs with simple cardiovascular stimulation

| *Agonist* | *Total dose (µg i.c.v.)* | *No. of responses* | *Arterial blood pressure increase (mmHg)* *Systolic* | *Diastolic* | *Heart rate increase (beats/min)* | *No. of cats* |
|---|---|---|---|---|---|---|
| Dopamine | 30 | 18 | 27.6 ± 3.1 | 25.4 ± 3.2 | 15.1 ± 3.6 | 6 |
| | 45 | 12 | 36.8 ± 4.0 | 30.7 ± 4.1 | 20.9 ± 3.8 | |
| α-Methyldopamine | 30 | 4 | 13.2 ± 1.7 | 11.0 ± 1.4 | 10.9 ± 2.1 | 1 |
| | 45 | 4 | 18.6 ± 1.9 | 17.2 ± 1.8 | 14.3 ± 1.9 | |

## Results

### Cardiovascular effects of i.c.v. dopamine and α-methyldopamine

The resting blood pressures and heart rates of the 10 animals used in this study were initially similar and remained virtually constant throughout the period of the study. Three control recordings of each cat were made on separate days and the group mean pressures were 114.3 ± 2.3 mmHg (systolic) and 82.6 ± 3.2 mmHg (diastolic) and the heart rate 139.7 ± 4.1 beats/minute.

Dopamine (30 and 45 μg i.c.v.) produced dose-related increases in blood pressure and heart rate in 6 cats and biphasic responses consisting of initial stimulant followed by prolonged depressor effects in the remaining 4. In those cats responding with pure stimulant effects the diastolic and systolic pressures were increased to a similar extent and the peak rises in blood pressure and heart rate occurred within 10 min of starting the drug infusion and had subsided within 30–45 min (Table 1). In those cats responding biphasically to i.c.v. dopamine the initial stimulant effects were over within 30 min of the infusion and were followed by depressor effects which reached a maximum 45–60 min post infusion with control levels of blood pressure and heart rate regained by 90 min (Table 2).

α-Methyldopamine (30 and 45 μg) was administered i.c.v. to 3 cats. In one cat it produced only stimulant effects on blood pressure and heart rate (Table 1) whilst in the remaining 2 it regularly produced biphasic responses (Table 2). The pressor and heart rate components of the responses to α-methyldopamine were smaller than those produced by the same dose of dopamine in each cat tested whilst the depressor effects were similar (Tables 1 and 2).

### Effect of autonomic ganglion blockade

Autonomic ganglion blockade was produced either by pempidine (5 to 7.5 mg/kg i.v.) or by hexamethonium (5 to 10 mg/kg). These dose levels were sufficient to abolish completely the pressor responses produced by the autonomic ganglion stimulant tetramethylammonium bromide (75 μg/kg i.v.). After ganglion blockade the pressor responses to i.c.v. dopamine and α-methyldopamine were abolished as were the secondary depressor components in those animals responding biphasically. Figure 1 illustrates an experiment in a cat which responded with a simple pressor response to i.c.v. dopamine (45 μg) with little change in heart rate. The pressor response was abolished 45 min after hexamethonium (10 mg/kg i.v.).

### Effect of adrenergic neurone blockade

The cardiovascular effects of i.c.v. dopamine (30 and 45 μg) were markedly reduced or abolished after

**Table 2** Maximal initial increases and secondary decreases in blood pressure and heart rate (mean ± s.e.) induced by two dose levels of each of dopamine and α-methyldopamine administered into the lateral cerebral ventricles of a group of conscious cats which responded biphasically to both drugs

| *Agonist* | *Total dose (μg i.c.v.)* | *No. of responses* | *Arterial blood pressure initial increase (mmHg)* | | *Heart rate initial increase (beats/min)* | *Arterial blood pressure secondary decrease (mmHg)* | | *Heart rate secondary decrease (beats/min)* | *No. of cats* |
|---|---|---|---|---|---|---|---|---|---|
| | | | *Systolic* | *Diastolic* | | *Systolic* | *Diastolic* | | |
| Dopamine | 30 | 12 | +23.1 ± 2.9 | +22.0 ± 3.1 | +14.2 ± 3.8 | −12.2 ± 2.8 | −12.7 ± 2.7 | −16.6 ± 3.0 | 4 |
| | 45 | 12 | +30.8 ± 3.2 | +29.5 ± 3.3 | +19.9 ± 3.9 | −18.6 ± 2.9 | −18.9 ± 3.0 | −23.2 ± 3.2 | |
| α-Methyldopamine | 30 | 8 | +10.6 ± 2.4 | + 9.9 ± 2.5 | +11.2 ± 3.1 | −11.1 ± 2.0 | −11.9 ± 2.3 | −13.7 ± 3.1 | 2 |
| | 45 | 8 | +19.3 ± 2.8 | +17.8 ± 3.5 | +15.9 ± 3.8 | −16.7 ± 3.3 | −17.0 ± 3.4 | −20.2 ± 4.0 | |

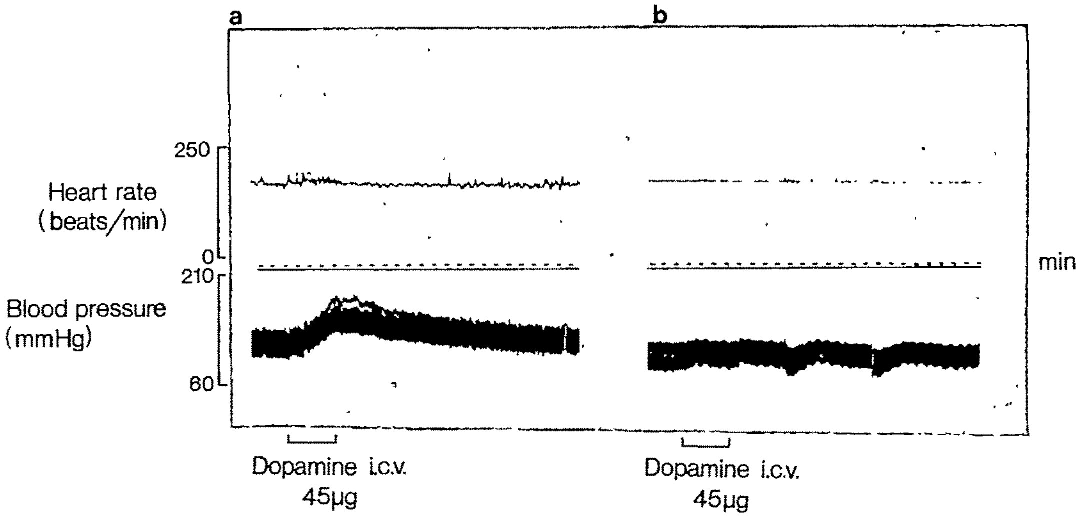


**Figure 1** Conscious cat; blood pressure and heart rate recordings. In (a) i.c.v. dopamine (45 µg) produced a large rise in blood pressure with little change in heart rate. This effect was abolished in (b) 45 min after ganglion blockade with hexamethonium (10 mg/kg, i.v.). Time-marker in this and subsequent figures is in one minute intervals.

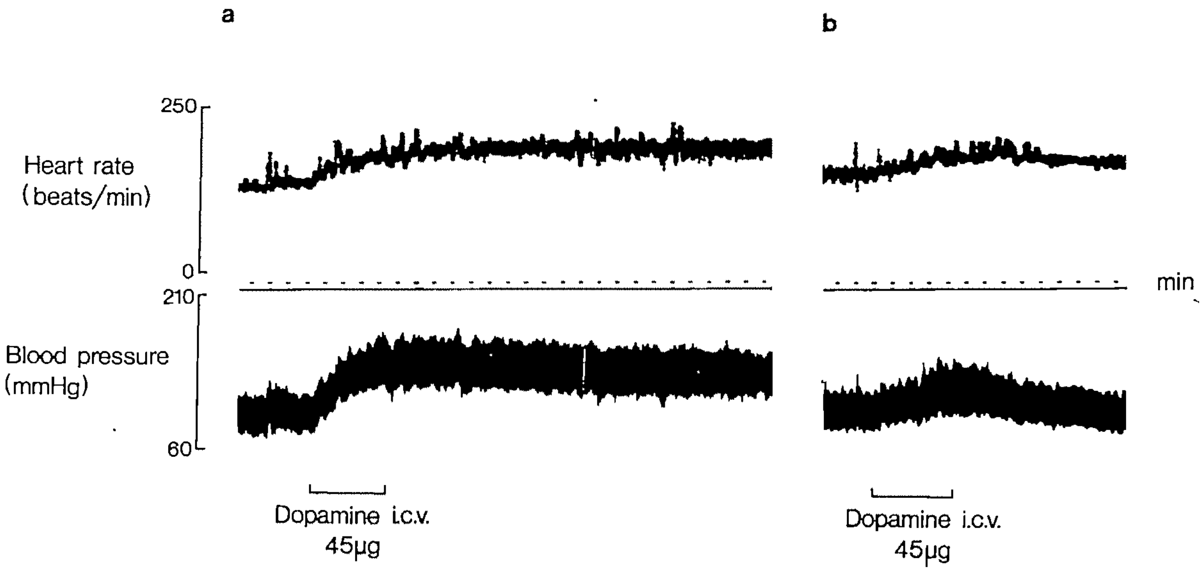


**Figure 2** Conscious cat; blood pressure and heart rate recordings. The pressor response and tachycardia produced by i.c.v. dopamine (45 µg) in (a) were markedly reduced in (b) 2 h after adrenergic neurone blockade with bethanidine (5 mg/kg i.v.)

adrenergic neurone blockade with bethanidine (5 mg/kg i.v.) in each of 10 cats tested. Figure 2 illustrates an experiment in which the pressor effect and tachycardia produced by i.c.v. dopamine (45 µg) were considerably reduced 2 h after the administration of bethanidine (5 mg/kg i.v.). Guanethidine was used in 2 cats (7.5 and 10 mg/kg i.v. respectively) and virtually abolished all the cardiovascular changes induced by i.c.v. dopamine.

In 3 cats adrenergic neurone blockade by

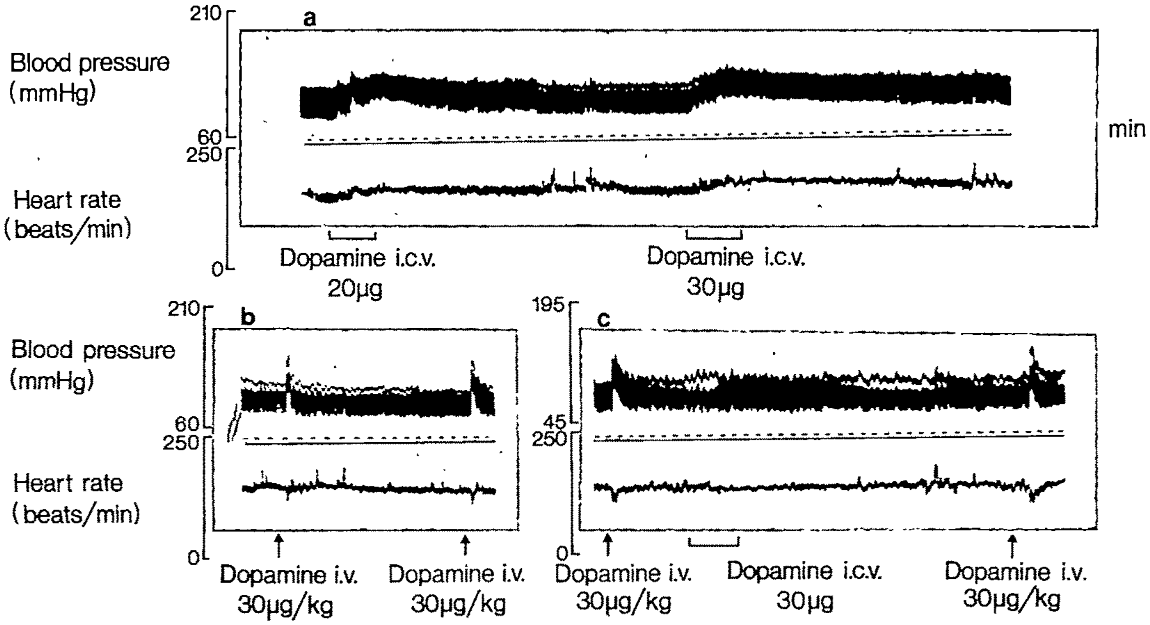


**Figure 3** Conscious cat; blood pressure and heart rate recordings. In (a) i.c.v. dopamine, at two dose levels, produced pressor responses associated with tachycardia. In (b) are shown the responses induced by i.v. dopamine. Between (b) and (c) (±)-propranolol (0.75 mg) was infused i.c.v. and panel (c) which commences 30 min later shows the unaltered responses to dopamine by the i.v. route and the abolition of responses to i.c.v. dopamine.

bethanidine or guanethidine produced a similar inhibition of the cardiovascular effects of i.c.v. $\alpha$-methyldopamine (45 μg).

### *Effect of i.c.v. pretreatment with $\beta$-adrenoceptor antagonists*

In the 6 cats which responded regularly with pressor effects and tachycardia to i.c.v. dopamine these effects were markedly inhibited 45–60 min after (±)-propranolol or (±)-alprenolol (0.5 to 1.0 mg, i.c.v.). These i.c.v. doses of $\beta$-adrenoceptor blockers have been shown previously to abolish completely the cardiovascular effects of i.c.v. isoprenaline in this preparation (Day & Roach, 1974a). Figure 3 illustrates the total blockade of the cardiovascular stimulant effects of i.c.v. dopamine (30 μg) by (±)-propranolol (0.75 mg, i.c.v.). In this experiment the pressor effects and associated bradycardias produced by intravenous dopamine (30 μg/kg) were unaffected by the central $\beta$-adrenoceptor blockade indicating that in this experiment the effects of both dopamine and propranolol after i.c.v. administration are confined to the central nervous system.

In 4 cats in which i.c.v. dopamine produced biphasic blood pressure responses both (±)-propranolol and (±)-alprenolol inhibited the initial pressor responses and associated tachycardias but did not affect the secondary hypotensions and bradycardias. These $\beta$-adrenoceptor blocking agents produced similar effects on the stimulant responses to i.c.v. $\alpha$-methyldopamine in 3 cats responding with simple cardiovascular stimulant effects and also in 2 others responding biphasically to this drug.

Administration of (+)-propranolol (0.25 to 0.5 mg i.c.v.) or (+)-alprenolol (0.25 to 0.75 mg) did not affect the cardiovascular effects of either dopamine or $\alpha$-methyldopamine administered i.c.v. Similarly, the local anaesthetics lignocaine (0.75 to 1 mg i.c.v.) and procaine (1.5 to 2 mg i.c.v.) were ineffective.

### *Effect of i.c.v. pretreatment with an $\alpha$-adrenoceptor antagonist*

In the 6 cats responding with cardiovascular stimulation to i.c.v. dopamine the administration of the $\alpha$-adrenoceptor antagonist phentolamine (0.5 to 0.75 mg i.c.v.) did not affect these responses. However, in the 4 cats responding biphasically to i.c.v. dopamine, i.c.v. phentolamine prevented the development of the secondary bradycardia and hypotension but did not affect the initial stimulant effects.

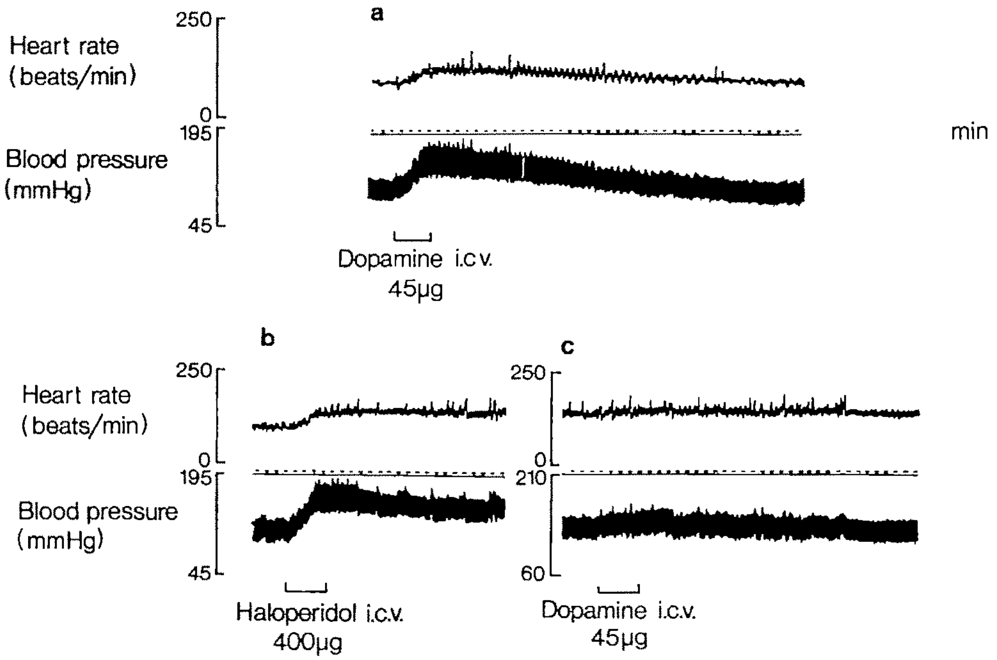


**Figure 4** Conscious cat; blood pressure and heart rate recordings. In (a), i.c.v. dopamine (45 µg) produced a large pressor response and tachycardia. (b) Haloperidol (400 µg i.c.v.) produced similar initial cardiovascular stimulation as did dopamine and 60 min later in (c) the effects of i.c.v. dopamine were abolished.

The responses to i.c.v. $\alpha$-methyldopamine were altered in the same way by i.c.v. phentolamine.

*Effect of central dopamine receptor blockade*

Haloperidol (400 µg, i.c.v.) produced a marked rise in blood pressure and heart rate in each of the 4 cats in which it was used. When these initial stimulant effects had subsided it was found that both stimulant and depressor effects of i.c.v. dopamine were abolished. These effects of haloperidol are illustrated in Figure 4. Since haloperidol was dissolved in an acid vehicle the effect of this alone was tested in 2 cats. The injection vehicle itself caused only a small pressor effect after i.c.v. infusion and did not affect the cardiovascular changes induced by i.c.v. dopamine.

Intravenous haloperidol (1 mg/kg) produced only slight cardiovascular stimulant effects itself but was as effective by this route as by i.c.v. administration in blocking both stimulant and depressor phases of the i.c.v. dopamine responses in two cats responding biphasically.

Pimozide (100 to 200 µg i.c.v.) produced much smaller cardiovascular stimulant effects than did i.c.v. haloperidol, control blood pressure and heart rate levels being regained after approximately 10 minutes. In 2 cats which responded with simple pressor responses to i.c.v. dopamine (45 µg) these effects were abolished 60 min after i.c.v. pimozide (200 µg). In 2 other cats which responded biphasically to i.c.v. dopamine the initial stimulant but not the secondary depressor phase of the response was abolished by i.c.v. pimozide. The vehicle in which pimozide was dissolved had no effects on the cardiovascular system which infused i.c.v. in similar volumes to those used for drug administration.

Intravenous pimozide (1 mg/kg) effectively inhibited the cardiovascular stimulant effects of i.c.v. dopamine (45 µg). Figure 5 illustrates an experiment with a cat which initially responded biphasically to the i.c.v. administration of dopamine. One hour after pimozide (1 mg/kg i.v.) the cardiovascular stimulant effects of i.c.v. dopamine (45 µg) were abolished, but the secondary hypotension and bradycardia still occurred.

*Effect of dopamine-$\beta$-hydroxylase inhibition*

Each of the 4 cats which responded biphasically to i.c.v. dopamine was pretreated for 3 days with the dopamine-$\beta$-hydroxylase inhibitor disulfiram (1st day 2 x 100 mg/kg i.p. separated by 12 h; 2nd and 3rd day 100 mg/kg i.p.). These cats were used 4 h after the final dose on the third day and it was found that

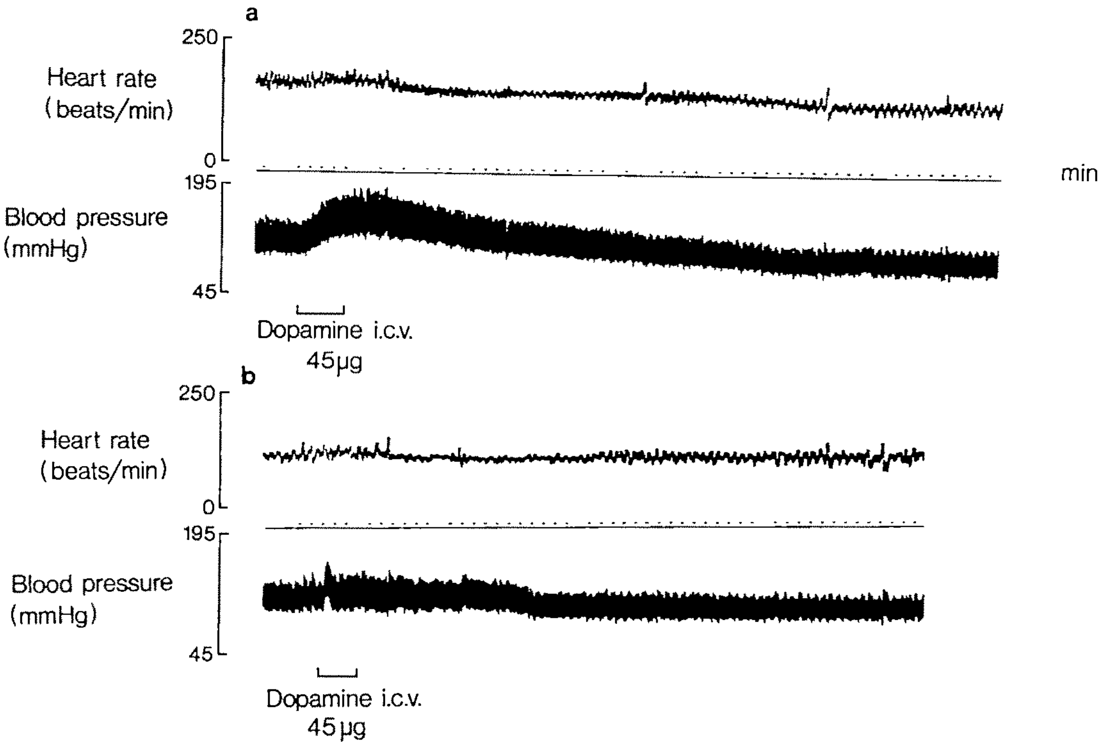


**Figure 5** Conscious cat; blood pressure and heart rate recordings. (a) Dopamine (45 µg i.c.v.) produced an initial rise in blood pressure and heart rate which later subsided into a hypotension with slight bradycardia. (b) The dopamine response repeated 60 min after i.v. pimozide (1 mg/kg); the initial stimulant effect of dopamine is absent but there is still a prolonged hypotensive response.

resting blood pressures and heart rates were unchanged from control levels. Dopamine (45 µg i.c.v.) produced slightly increased initial pressor responses and tachycardias in these animals compared with control responses. However, the secondary falls in blood pressure and heart rate normally occurring in these animals were absent after disulfiram. α-Methyldopamine (30 µg i.c.v.) was tested in one of these animals and, like dopamine, was found to produce only cardiovascular stimulation.

### *Comparison of the cardiovascular effects of dopamine and isoprenaline by i.c.v. administration*

Since it appeared that β-adrenoceptors may be involved in the cardiovascular responses produced by i.c.v. dopamine, the cardiovascular effects produced by i.c.v. administration of this substance were compared with those produced by isoprenaline in the 10 cats used in the present study. In the 6 cats which responded to i.c.v. dopamine with simple cardiovascular stimulation 4 responded to i.c.v. isoprenaline (30 µg) with pressor responses and tachycardia whilst in the other 2 there was a fall in blood pressure associated with tachycardia. Of the 4 cats which responded biphasically to i.c.v. dopamine, isoprenaline (30 µg i.c.v.) produced pressor responses with associated tachycardia in two, hypotension with tachycardia in one and tachycardia without blood pressure change in the last animal. Pressor, depressor and heart rate changes induced by i.c.v. isoprenaline were all abolished by (±)-propranolol (0.5 to 1 mg i.c.v.).

Neither pimozide (1 mg/kg i.v.) nor haloperidol (1 mg/kg i.v.) affected the cardiovascular changes induced by i.c.v. isoprenaline.

### *Effect of dopamine administered into the cisterna magna (i.c.m.)*

Two cats previously used for i.c.v. administration of dopamine were additionally cannulated to allow of drug infusion into the cisterna magna. One cat responded to i.c.v. dopamine with a simple pressor response and tachycardia whilst the other responded biphasically. Infusion into the cisterna magna of dopamine (30 and 45 µg) produced dose-related falls in blood pressure accompanied by bradycardia in each cat.

However, a higher dose (75 µg) of dopamine by this route produced an initial pressor response and tachycardia followed by prolonged hypotension and bradycardia in each cat. This higher dose of dopamine usually induced vomiting by the i.c.m. route which it never did i.c.v. The mean initial stimulant and subsequent depressor effects of the 75 µg i.c.m. dose of dopamine are summarized in Table 3. Phentolamine (0.5 mg i.c.m.) did not affect resting blood pressure or heart rate, but it abolished the inhibitory effects of the lower dopamine doses and also the inhibitory component of the response to the highest dopamine dose without significantly affecting the pressor component of the response. In one experiment dopamine did not induce vomiting following phentolamine suggesting that the pressor effect of dopamine by this route is not secondary to the stress of vomiting.

The initial pressor but not the secondary depressor effects of i.c.m. dopamine (75 µg) were absent 1.5 h after i.c.m. administration of (±)-propranolol (0.5 mg) in each cat. Neither cat vomited in response to i.c.m. dopamine after (±)-propranolol treatment.

**Table 3** Maximal initial increases and secondary decreases in blood pressure and heart rate (mean ± s.e.) induced by 75 µg dopamine infused into the cisterna magna (i.c.m.) of two conscious cats

| *Agonist* | *Total dose (µg i.c.m.)* | *No. of responses* | *Arterial blood pressure initial increase (mmHg)* | | *Heart rate initial increase (beats/min)* | *Arterial blood pressure secondary decrease (mmHg)* | | *Heart rate secondary decrease (beats/min)* | *No. of cats* |
|---|---|---|---|---|---|---|---|---|---|
| | | | *Systolic* | *Diastolic* | | *Systolic* | *Diastolic* | | |
| Dopamine | 75 | 4 | +20.1±5.9 | +18.8±6.1 | +27.1±5.2 | −24.8±4.5 | −25.3±4.7 | −31.7±5.6 | 2 |

## Discussion

The central administration of dopamine and its $\alpha$-methyl analogue to several species has been reported to produce falls in arterial blood pressure with associated bradycardia (McCubbin *et al.*, 1960; Baum & Shropshire, 1973; Heise & Kroneberg, 1973; Bloch *et al.*, 1974; Finch *et al.*, 1975). Heise & Kroneberg (1973), using anaesthetized cats reported that the hypotension in response to i.c.v. $\alpha$-methyldopamine was blocked by i.c.v. phentolamine or yohimbine although that to i.c.v. dopamine was relatively unaffected by i.c.v. phentolamine. Finch *et al.* (1975) analysed the mechanism of hypotension produced by i.c.v. $\alpha$-methyldopamine in conscious spontaneously hypertensive rats. They reported that the hypotension induced by i.c.v. $\alpha$-methyldopamine was prevented by i.c.v. phentolamine or desmethylimipramine but not by systemic haloperidol. Inhibition of central dopamine $\beta$-hydroxylase abolished the depressor effect of i.c.v. $\alpha$-methyldopamine. These data suggest that in the rat i.c.v. $\alpha$-methyldopamine produces hypotension by acting as a precursor for the formation of $\alpha$-methylnoradrenaline which stimulates inhibitory $\alpha$-adrenoceptors within the brain (van Zwieten, 1973). However, Finch *et al.* (1975) did not produce corroborative data for this hypothesis from their work in anaesthetized cats since, in their experiments, both noradrenaline and $\alpha$-methylnoradrenaline by the i.c.v.

route produced increases in blood pressure, results in direct conflict with previous reports in conscious cats (Day & Roach, 1973; 1974a).

The mechanisms whereby the dopamine receptor stimulant apomorphine produces its cardiovascular responses are similarly confused. Barnett & Fiore (1971) reported that apomorphine lowered blood pressure in anaesthetized cats and that this effect was abolished by either spinal section or haloperidol treatment. These workers concluded that the hypotensive effect of apomorphine was due to stimulation of central dopamine receptors. However, Finch & Haeusler (1973) could not abolish the hypotensive effects of apomorphine in rats with haloperidol, spiroperidol or sulpiride, all substances reported to possess potent dopamine receptor blocking activity. Finch & Haeusler (1973) suggested that the hypotensive response to apomorphine in their experiments was due to increased efferent vagal activity and was not mediated through activation of central dopamine receptors.

In the systemic cardiovascular system dopamine has been reported to stimulate $\alpha$ and $\beta$-adrenoceptors and dopamine receptors (Goldberg & Whitsett, 1971). Intracerebroventricular dopamine in the present experiments caused increases in blood pressure with associated tachycardia in each of the 10 cats used. These cardiovascular stimulant effects were apparently mediated via the peripheral sympathetic system since they were absent after either ganglion blockade or adrenergic neurone blockade. Moreover, the effects of i.c.v. dopamine were abolished by i.c.v. $\beta$-adrenoceptor blockers or by i.c.v. dopamine antagonists. In 4 of the cats used, after the initial stimulant effects of i.c.v. dopamine had subsided they were replaced by a prolonged fall in both blood pressure and heart rate. These secondary depressor effects of i.c.v. dopamine were abolished by phentolamine and by disulfiram. Disulfiram has been shown to be an effective inhibitor of central dopamine $\beta$-hydroxylase (Musacchio, Goldstein, Anagnoste, Poch & Kopin, 1966). Thus it would appear that in these cats some of the i.c.v. dopamine was converted to noradrenaline which then produced typical centrally-mediated cardiovascular depressant effects (Day & Roach, 1974a).

Haloperidol inhibited all the cardiovascular changes induced by i.c.v. dopamine whilst pimozide only inhibited the stimulant effects. These results are not unexpected considering the published data on these compounds. Thus, haloperidol is less specific than pimozide and blocks both noradrenaline and dopamine receptors in the central nervous systems of rats (Carlsson & Lindqvist, 1963), cats and dogs (Janssen, 1967), whilst pimozide has been reported to block only dopamine receptors in rats, cats and dogs (Janssen, 1967; Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970).

Bolme & Fuxe (1971) reported that in the cat, spiroperidol and pimozide caused hypotension and bradycardia which were potentiated by clonidine. They suggested that these effects might have been mediated via removal of excitatory central dopaminergic tone. They further postulated the existence in the brain of opposing dopaminergic and noradrenergic mechanisms influencing the peripheral cardiovascular system. Contrasting data were reported by Bloch *et al.* (1974) who found that clonidine and large doses of dopamine applied to the ventral surface of the cat brain stem induced hypotension. The effects of each compound were antagonized by pimozide whilst noradrenaline applied to this area caused pressor responses. In the present experiments i.c.v. haloperidol produced pronounced cardiovascular stimulant effects. It is not clear what the mechanism of this effect is; it could be due to initial dopamine receptor stimulant activity, or conversely, to inhibition of noradrenergic depressant pathways. It was found in the present experiments and in those reported previously (Day & Roach, 1974a) that i.c.v. phentolamine caused increases in heart rate and blood pressure. Pimozide which is a more specific dopamine receptor antagonist produced only slight and transient cardiovascular stimulation after i.c.v. administration. The results obtained with two cats in which dopamine was administered into the cisterna magna suggested that its predominant effect at this site was to cause cardiovascular depressant effects, apparently mediated via formation of noradrenaline. Thus it would appear that the increase in blood pressure and heart rate produced by i.c.v. dopamine were caused by stimulation of supramedullary structures.

Both dopamine and isoprenaline produce cardiovascular stimulant effects after i.c.v. infusion in conscious cats (Day & Roach, 1974a). These effects of both drugs are abolished by $\beta$-adrenoceptor blocking agents and tempt the speculation that both are mediated via the same receptors. However, the evidence from the present experiments, although not inconsistent with this possibility, lends it little support. Thus, although there was considerable variation in the responses to isoprenaline and dopamine in different cats the response to each agent remained constant within individual cats and qualitative differences in the responses were regularly found. Isoprenaline produced a more marked rise in systolic than diastolic pressure whilst dopamine increased both equally. Furthermore the pressor responses to i.c.v. isoprenaline were always accompanied by more marked tachycardias than were similar pressor responses to dopamine. These observations are not inconsistent with a similar action of dopamine and isoprenaline on blood pressure regulating centres with the possibility of a more marked effect of isoprenaline on centres controlling heart rate. The fact that pimozide selectively abolished dopamine responses suggests that the actual receptors mediating blood pressure responses to dopamine and isoprenaline are different but does not preclude the possibility that a common neural pathway, possibly terminating in β-adrenoceptors, is involved in both responses.

The published evidence concerning the function of brain noradrenaline in cardiovascular control suggests that its role is inhibitory to the sympathetic outflow (see reviews by van Zwieten, 1973; Day & Roach, 1974b). The report of decreased noradrenaline synthesis and content of the brain stem and hypothalamus of spontaneously hypertensive rats (Yamori, Lovenberg & Sjoerdsma, 1970) supports this concept as does the finding of decreased turnover rate of noradrenaline in the brain stem of DOCA/saline hypertensive rats (Nakamura, Gerold & Thoenen, 1971).

Yamabe, de Jong & Lovenberg (1973) reported increased dopamine levels in the telencephalon of spontaneously hypertensive rats whilst Spring & Winkelmuller (1975) have recently produced evidence in favour of dopamine participation in the pressor response of anaesthetized cats to electrical stimulation of the ventral mid-brain. These observations together with those presented here are consistent with a role for brain dopamine as a mediator concerned with cardiovascular stimulation via activation of the peripheral sympathetic nervous system.

The authors are grateful to Merck, Sharpe and Dohme Ltd., West Point, Pennsylvania, U.S.A. for a gift of (–)-methyldopamine and to the Medical Research Council for a training award to one of us (A.G.R.).

## References

ANDÉN, N.E., BUTCHER, S.G., CORRODI, H., FUXE, K. & UNGERSTEDT, U. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmac.*, **11**, 303–314.

ARDILL, B.L., FENTEM, P.H. & WELLARD, M.J. (1967). An electromagnetic pressure generator for testing the frequency response of transducers and catheter systems. *J. Physiol., Lond.*, **192**, 19–21P.

BARNETT, A. & FIORE, J.W. (1971). Hypotensive effects of apomorphine in anaesthetised cats. *Eur. J. Pharmac.*, **14**, 206–208.

BAUM, T. & SHROPSHIRE, A.T. (1973). Reduction of sympathetic outflow by central administration of L-dopa, dopamine and norepinephrine. *Neuropharmacology*, **12**, 49–56.

BLOCH, R., BOUSQUET, P., FELDMAN, J., VELLY, J. & SCHWARTZ, J. (1974). Action hypotensive de la clonidine appliquée sur la surface ventrale du bulbe rachidien: analogie avec la dopamine. *Thérapie*, **29**, 251–259.

BOLME, P. & FUXE, K. (1971). Pharmacological studies on the hypotensive effects of clonidine. *Eur. J. Pharmac.*, **13**, 168–174.

CARLSSON, A. & LINDQVIST, M. (1963). Effect of chlorpromazine or haloperidol on the formation of 3-methoxytryptamine and normetanephrine in mouse brain. *Acta pharmac. tox.*, **20**, 140–144.

COOLING, M.J., DAY, M.D. & ROACH, A.G. (1974). A modified cannula for intracerebroventricular injections in conscious cats. *Lab. Pract.*, **23**, 439.

DAY, M.D. & ROACH, A.G. (1972). Adrenoceptors in the central nervous system concerned with cardiovascular control. *J. Pharm. Pharmac.*, **24**, 148P.

DAY, M.D. & ROACH, A.G. (1973). β-Adrenergic receptors in the central nervous system of the cat concerned with control of arterial blood pressure and heart rate. *Nature, New Biol.*, **242**, 30–31.

DAY, M.D. & ROACH, A.G. (1974a). Central α- and β-adrenoceptors modifying arterial blood pressure and heart rate in conscious cats. *Br. J. Pharmac.*, **51**, 325–333.

DAY, M.D. & ROACH, A.G. (1974b). Central adrenoceptors and the control of arterial blood pressure. *Clin. exp. Pharm. Physiol.*, **1**, 347–360.

DAY, M.D. & WHITING, R.L. (1972). An improved valve device for the continuous measurement of arterial blood pressure in the conscious unrestrained cat. *J. Pharm. Pharmac.*, **24**, 263–264.

DE JONG, W., NIJKAMP, F.P. & BOHUS, B. (1975). Role of noradrenaline and serotonin in the central control of blood pressure in normotensive and spontaneously hypertensive rats. *Arch. int. Pharmacodyn. Thér.*, **213**, 272–284.

FELDBERG, W., MYERS, R.D. & VEALE, W.L. (1970). Perfusion from central ventricle to cisterna magna in the unanesthetised cat. Effect of calcium on body temperature. *J. Physiol., Lond.*, **207**, 403–416.

FELDBERG, W. & SHERWOOD, S.L. (1953). A permanent cannula for intraventricular injections in cats. *J. Physiol., Lond.*, **120**, 3–5P.

FINCH, L. & HAEUSLER, G. (1973). The cardiovascular effects of apomorphine in the anaesthetised rat. *Eur. J. Pharmac.*, **21**, 264–270.

FINCH, L., HERSOM, A. & HICKS, P. (1975). Studies on the hypotensive action of α-methyldopamine. *Br. J. Pharmac.*, **54**, 445–452.

GOLDBERG, L.I. & WHITSETT, T.L. (1971). Cardiovascular effects of levodopa. *J. Am. Med. Ass.*, **218**, 1921–1923.

HEISE, A. & KRONEBERG, G. (1973). Central nervous α-adrenergic receptors and the mode of action of α-methyldopa. *Naunyn-Schmiedebergs Arch. Pharmac.*, **279**, 285–300.

HENNING, M. (1973). Receptor regulation studies: significance of central catecholamine receptors in cardiovascular control. In *Frontiers in Catecholamine Research*, ed. Usdin, E. & Snyder, S., pp. 595–600. New York: Pergamon Press.

HENNING, M. (1975). Central sympathetic transmitters and hypertension. *Clin. Sci. Mol. Med.*, **48**, 195–203S.

JANSSEN, P.A.J. (1967). Haloperidol and related butyrophenones. In *Psychopharmacological Agents*, Vol. II, ed. Gordon, M., pp. 199–248. New York, London: Academic Press.

McCUBBIN, J.W., KANEKO, Y. & PAGE, I.H. (1960). Ability of serotonin and norepinephrine to mimic the central effects of reserpine on vasomotor activity. *Circulation Res.*, **8**, 849–858.

MUSACCHIO, J.M., GOLDSTEIN, M., ANAGNOSTE, B., POCH, G. & KOPIN, I.J. (1966). Inhibition of dopamine-$\beta$-hydroxylase by disulfiram *in vivo*. *J. Pharmac. exp. Ther.*, **152**, 56–61.

NAKAMURA, K., GEROLD, M. & THOENEN, H. (1971). Experimental hypertension of the rat: Reciprocal changes of norepinephrine turnover in heart and brainstem. *Naunyn-Schmiedebergs Arch. Pharmac.*, **268**, 125–139.

SPRING, A. & WINKELMULLER, W. (1975). Ventral midbrain stimulation, blood pressure responses and their relation to the dopaminergic nigro-striatal pathways. *Pflügers Arch. Eur. J. Physiol.*, **358**, 339–348.

STRUYKER BOUDIER, H.A.J., SMEETS, G.W.M., BROUWER, G.M. & VAN ROSSUM, J.M. (1974). Hypothalamic alpha adrenergic receptors in cardiovascular regulation. *Neuropharmacology*, **13**, 837–846.

STRUYKER BOUDIER, H.A.J., SMEETS, G.W.M., BROUWER, G.M. & VAN ROSSUM, J.M. (1975). Central nervous system $\alpha$-adrenergic mechanisms and cardiovascular regulation in rats. *Arch. int. Pharmacodyn. Thér.*, **213**, 285–293.

TOIVOLA, P. & GALE, C.C. (1970). Effect on temperature of biogenic amine infusion into hypothalamus of baboon. *Neuroendocrinology*, **6**, 210–219.

VAN ZWIETEN, P.A. (1973). The central action of antihypertensive drugs, mediated via central $\alpha$-receptors. *J. Pharm. Pharmac.*, **25**, 89–95.

YAMABE, H., DE JONG, W. & LOVENBERG, W. (1973). Further studies on catecholamine synthesis in the spontaneously hypertensive rat: Catecholamine synthesis in the central nervous system. *Eur. J. Pharmac.*, **22**, 91–98.

YAMORI, Y., LOVENBERG, W. & SJOERSDMA, A. (1970). Norepinephrine metabolism in brain stem of spontaneously hypertensive rats. *Science*, **170**, 544–546.

*(Received February 20, 1976.*
*Revised May 13, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 517–520

# EFFECT OF MORPHINE AND NALOXONE ON PRIMING-INDUCED AUDIOGENIC SEIZURES IN BALB/c MICE

C.S. CHEN & G.R. GATES
Department of Psychology, Monash University, Clayton, Victoria, Australia
J.A. REYNOLDSON
Department of Pharmacology, Monash University, Clayton, Victoria, Australia

**1** Morphine (1–200 mg/kg, s.c.) reduced the incidence and prolonged the latency of priming-induced audiogenic seizures in a dose-dependent manner.

**2** This effect was reversed by naloxone (1 and 2 mg/kg) although naloxone was itself inactive.

**3** This priming-induced seizure model may be useful in the study of tolerance and physical dependence.

## Introduction

Many drugs have been shown to suppress the incidence and severity of audiogenic seizures (that is, seizures induced by a loud sound). These seizures can be suppressed by centrally acting drugs such as tranquillizers (Plotnikoff, 1960) and sedatives (Collins & Horlington, 1969). However, little has been done to investigate the effects of morphine and its antagonism by naloxone in this test. Morphine has been investigated by Collins & Horlington (1969) and Boggan (1973) and apomorphine has been shown to suppress audiogenic seizures (Anlezark & Meldrum, 1975).

Seizure-resistant strains of mice can be made reliably and consistently seizure-prone, by comparison with spontaneously seizure-prone mice, by priming procedures (SJL/J mice: Fuller & Collins, 1968; CF-1 mice: Iturrian & Fink, 1968; BALB/c mice: Henry & Bowman, 1969; heterogeneous strain: Boggan, Freedman & Lovell, 1971; Gates & Chen, 1976) and thus the priming procedure (as described below) may facilitate pharmacological studies (Iturrian & Johnson, 1970).

The aim of this study was to test morphine and its antagonist naloxone using priming-induced audiogenic seizures.

## Methods

Twenty-one day old (±1 day) BALB/c mice were primed by 25 s exposure to a bell of intensity 131 dB re 0.0002 dyn $cm^{-2}$ and tested for seizures 9 days (±1 day) later by re-exposure to the same acoustic stimulus for a maximum of 60 s or until seizure occurred. The parameters for priming in this line of mice have been described elsewhere (Chen; 1973). The incidence of four stages of audiogenic seizures was recorded (wild running, clonic seizure, tonic seizure and death) as were the latencies of onset of the first two stages.

Control injections and drugs were administered on a split litter basis. In the first experiment the effects of saline, naloxone and morphine were tested alone. Saline (0.9% w/v NaCl solution) or morphine was given 20 min before testing and 15 mice were used at each dose level (1, 2.5, 7.5, 25, 50, 100 and 200 mg/kg) of morphine. Naloxone was given to 20 mice at each of four doses (1, 5, 10 and 20 mg/kg), 20 min before testing. In the second experiment the effect of the combination of morphine and naloxone given 20 min before testing was studied. Four doses of morphine (7.5, 50, 200, 400 mg/kg) were tested alone and in combination with 1 and 2 mg/kg of naloxone. Each group contained 20 mice.

## Drugs

Morphine hydrochloride (McFarlane Smith) and naloxone hydrochloride (Bristol Laboratories) were used. All drugs were given subcutaneously in a volume of 0.1 ml per 10 g weight.

## Results

As saline-injected mice showed no significant difference from those not injected, in each of the morphine and naloxone experiments these groups were pooled to form control groups with which drug effects were compared.

In the first experiment naloxone was found to be ineffective in altering the incidence of any audiogenic seizure component (Fisher Exact test, $P > 0.1$ in each case, two tailed, Table 1) or in altering the latencies of wild running (Kruskal-Wallis, $H(3) = 1.605$, $P > 0.5$) or of clonic seizure (Kruskal-Wallis, $H(3) = 6.539$, $P > 0.05$). However, morphine reduced the incidence of all seizure components and prolonged the latency of wild running (Kruskal-Wallis $H(6) = 24.618$, $P < 0.001$) and clonic seizure (Kruskal-Wallis, $H(6) = 27.484$, $P < 0.001$). The suppression by morphine was dose-dependent and the lowest dose which significantly reduced clonic seizures was 1 mg/kg (Fisher Exact test, $P < 0.05$, two tailed). The lowest dose which significantly reduced wild running, clonic and tonic seizures was 2.5 mg/kg (Fisher Exact test, $P < 0.005$, two tailed) while all higher doses significantly reduced wild running and clonic seizures and completely inhibited the tonic seizure stage (Table 1). The initially low incidence of death (20%) was not significantly altered by morphine although doses of 7.5 mg/kg and above completely abolished it. However, it was impossible to abolish completely either wild running or clonic seizure, and the effect of morphine reached a maximum at 50 mg/kg, when these seizure stages were reduced from 100% to 33.3% and 26.7% respectively.

The results of the second experiment as shown in Figure 1 indicate the antagonistic effect of naloxone on the suppression of audiogenic seizures by morphine. At all doses of morphine, 2 mg/kg of naloxone produced a significant reversal of suppression of the first three stages of audiogenic seizures to a level not significantly different from that in mice treated with naloxone alone.

Naloxone, at a dose of 1 mg/kg, significantly antagonized the suppressive effects of 7.5, 50 and 400 mg/kg of morphine on wild running, clonic and tonic seizure (see Figure 1, Fisher Exact test, $P < 0.05$ in each case). However, naloxone, at this dose, did not significantly antagonize the effect of 200 mg/kg of morphine. This result is difficult to interpret but may be related to the well known biphasic excitant-depressant action of morphine (Tatum, Seevers & Collins, 1929) and the ability of morphine to cause convulsions in some species at high doses (Hazelton & Koppanyi, 1941). Although the low incidence of death in these animals prevented the use of this factor in analysis, 1 mg/kg of naloxone did produce a significant reversal of the protective action of 7.5 mg/kg of morphine (Fisher Exact test, $P < 0.01$, two tailed). Similarly 2 mg/kg of naloxone significantly reversed the effect of 50 mg/kg of morphine on death (Fisher Exact test, $P < 0.05$, two tailed).

Analysis of the latencies of wild running and clonic seizures indicates that, irrespective of the presence of naloxone, morphine causes a dose-dependent significant increase in the latency of these parameters (Kruskal-Wallis, W.R., $H(3) = 24.496$, $P < 0.001$; clonic seizure, $H(3) = 26.176$, $P < 0.001$). Naloxone

**Table 1** Effect of naloxone and morphine on the incidence of audiogenic seizures in BALB/c mice

| *Treatment (doses in mg/kg)* | n | *Incidence of seizure component* W | C | T | D |
|---|---|---|---|---|---|
| Control | 40 | 39 | 33 | 20 | 9 |
| Naloxone | | | | | |
| 1 | 20 | 17 | 15 | 11 | 8 |
| 5 | 20 | 17 | 13 | 10 | 8 |
| 10 | 20 | 20 | 18 | 9 | 7 |
| 20 | 20 | 19 | 13 | 7 | 5 |
| Control | 40 | 40 | 40 | 28 | 8 |
| Morphine | | | | | |
| 1 | 15 | 12 | 11* | 8 | 5 |
| 2.5 | 15 | 11* | 11* | 2* | 1 |
| 7.5 | 15 | 11* | 8* | 0* | 0 |
| 25.0 | 15 | 6* | 4* | 0* | 0 |
| 50.0 | 15 | 5* | 4* | 0* | 0 |
| 100.0 | 15 | 7* | 6* | 0* | 0 |
| 200.0 | 15 | 8* | 6* | 0* | 0 |

W: wild running; C: clonic seizure; T: tonic seizure; D: death.
Asterisks indicate which groups, when compared with controls using a two-tailed Fisher Exact test, were significantly different at the 5% probability level.

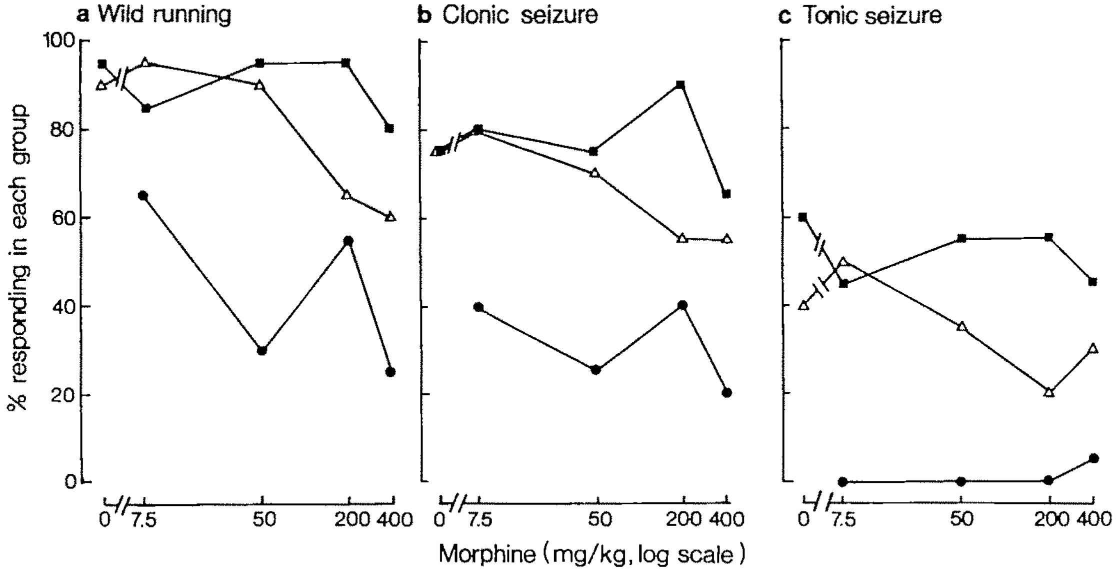


**Figure 1** The effect of morphine, and its antagonism by naloxone, on the incidence of three phases of the audiogenic seizure response in BALB/c mice. Naloxone reduced the effect of morphine in a dose-dependent manner, 2 mg/kg returning the incidence of seizure to control levels. (●) Morphine; (△) morphine + 1 mg/kg naloxone; (■) morphine + 2 mg/kg naloxone.

does not significantly antagonize the effects of morphine on latency of wild running and clonic seizure. However, the median latencies of wild running were 4.1, 3.85 and 3.6 s for the morphine, morphine plus naloxone 1 mg/kg, morphine plus naloxone 2 mg/kg groups, respectively (Kruskal-Wallis, $H(2)=2.931$, $P>0.2$). There was a similar trend in the latency of clonic seizure, the medians being 8.5, 8.4 and 7.25 s respectively (Kruskal-Wallis, $H(2)=2.474$, $P>0.2$).

## Discussion

Our results show that morphine can suppress the incidence and severity of priming-induced audiogenic seizures in normally seizure-resistant BALB/c mice (Chen, 1973). Naloxone can reverse this suppression of seizures by morphine and thus suggests that morphine is acting via a specific receptor.

The results presented here differ from the negative findings of Bentley (1961) who used rats, and Collins & Horlington (1969) who used a spontaneously susceptible strain of mice and measured only complete abolition of the wild running component. Similarly Boggan (1973) found that morphine did not suppress priming-induced seizures in C57 BL/6 mice. This discrepancy between C57 BL/6 mice used by Boggan and our results cannot be accounted for by dosage differences but may be due to strain differences as there is some evidence for different strain susceptibility to morphine (Gebhart & Mitchell, 1973; Oliverio & Castellano, 1974).

This seizure test may provide a useful model for the study of tolerance and physical dependence development, particularly since it has been shown that alcohol (Freund & Walker, 1971) and barbiturate (Gates & Chen, 1974; Bentley, 1961) withdrawal can induce seizures in some resistant strains of mice and rats.

We are indebted to Dr G.A. Bentley for his helpful criticism of the manuscript. This study was supported by an Australian Research Grants Committee grant to C-S.C.

## References

ANLEZARK, G.M. & MELDRUM, B.S. (1975). Effects of apomorphine, ergocornine and piribedil on audiogenic seizures in DBA/2 mice. *Br. J. Pharmac.*, **53**, 419–421.

BENTLEY, G.A. (1961). The susceptibility of rats to audiogenic seizures following acute and prolonged medication with narcotic drugs. *Arch. int. Pharmacodyn.*, **132**, 378–391.

BOGGAN, W.O. (1973). Psychoactive compounds and audiogenic seizure susceptibility. *Life Sci.*, **13**, 151–159.

BOGGAN, W.O., FREEDMAN, D. & LOVELL, R.A. (1971). Study in audiogenic seizure susceptibility. *Psychopharmacologia, (Berl)*, **20**, 48–56.

CHEN, C-S. (1973). Sensitization for audiogenic seizures in two strains of mice and their $F_1$ hybrids. *Devel. Psychobiol.*, **6**, 131–138.

COLLINS, A.J. & HORLINGTON, M. (1969). A sequential screening test based on the running component of audiogenic seizures in mice, including reference compound PD50 values. *Br. J. Pharmac.*, **37**, 140–150.

FREUND, G. & WALKER, D.W. (1971). Sound-induced seizures during ethanol withdrawal in mice. *Psychopharmacologia, (Berl)*, **22**, 45–49.

FULLER, J.L. & COLLINS, R.L. (1968). Temporal parameters of sensitization for audiogenic seizures in SJL/J mice. *Devel. Psychobiol.*, **1**, 185–188.

GATES, G.R. & CHEN, C-S. (1974). Effects of barbiturate withdrawal on audiogenic seizure susceptibility in BALB/c mice. *Nature, Lond.*, **249**, 162–164.

GATES, G.R. & CHEN, C-S. (1976). Priming for audiogenic seizures in BALB/c mice as a function of stimulus exposure duration and age. *Exp. Neurol.*, **51**, 593–602.

GEBHART, G.F. & MITCHELL, C.L. (1973). Strain differences in the analgesic response to morphine as measured on the hot plate. *Arch. int. Pharmacodyn.*, **201**, 128–140.

HAZELTON, L.W. & KOPPANYI, T. (1941). The effect of central stimulants in experimental morphine poisoning in rabbits. *Anaesthesiology*, **2**, 427–442.

HENRY, K.R. & BOWMAN, R.E. (1970). Acoustic priming of audiogenic seizures in mice. In *Physiological Effects of Noise*. ed. Welch, B.L. & Welch, A.S. New York: Plenum Press.

ITURRIAN, W.B. & FINK, G.B. (1968). Effects of age and condition-test interval (days) on an audioconditioned convulsive response in CF-1 mice. *Devel. Psychobiol.*, **1**, 230–235.

ITURRIAN, W.B. & JOHNSON, H.D. (1970). Audio-sensitization; a potential screening method for drugs affecting the CNS. *J. Pharmac. Sci.*, **59**, 1046–1047.

OLIVERIO, A. & CASTELLANO, C. (1974). Genotype-dependent sensitivity and tolerance to morphine and heroin: Dissociation between opiate-induced running and analgesia in the mouse. *Psychopharmacologia, (Berl)*, **39**, 13–22.

PLOTNIKOFF, N. (1960). Ataractics and strain differences in audiogenic seizures in mice. *Psychopharmacologia (Berl).*, **1**, 429–432.

TATUM, A.L., SEEVERS, M.H. & COLLINS, K.H. (1929). Morphine addiction and its physiological interpretation based on experimental evidence. *J. Pharmac. exp. Ther.*, **36**, 447–475.

*(Received March 9, 1976.*
*Revised May 14, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 521–527

# LONG-TERM EFFECTS OF *N*-2-CHLOROETHYL-*N*-ETHYL-2-BROMOBENZYLAMINE HYDROCHLORIDE ON NORADRENERGIC NEURONES IN THE RAT BRAIN AND HEART

S.B. ROSS

Research and Development Laboratories, Astra Läkemedel AB, S-151 85 Södertälje, Sweden

1 *N*-2-Chloroethyl-*N*-ethyl-2-bromobenzylamine hydrochloride (DSP 4) 50 mg/kg intraperitoneally, produced a long-term decrease in the capacity of brain homogenates to accumulate noradrenaline with significant effect 8 months after the injection. It had no effect on the noradrenaline uptake in homogenates from the striatum (dopamine neurones) and on the uptake of 5-hydroxytryptamine (5-HT) in various brain regions.

2 *In vitro* DSP 4 inhibited the noradrenaline uptake in a cortical homogenate with an $IC_{50}$ value of 2 μM but was more than ten times less active on the dopamine uptake in a striatal homogenate and the 5-HT uptake in a cortical homogenate.

3 DSP 4 (50 mg/kg i.p.) inhibited the uptake of noradrenaline in the rat heart atrium *in vitro* but this action was terminated within 2 weeks.

4 DSP 4 (50 mg/kg i.p.) caused a decrease in the dopamine-β-hydroxylase (DBH) activity in the rat brain and heart. The onset of this effect was slow; in heart a lag period of 2–4 days was noted. In brain the DBH-activity in cerebral cortex was much more decreased than that in hypothalamus which was only slightly affected. A significant effect was still found 8 months after the injection. The noradrenaline concentration in the brain was greatly decreased for at least two weeks, whereas noradrenaline in heart was only temporarily reduced.

5 The long-term effects of DSP 4 on the noradrenaline accumulation, the DBH activity and noradrenaline concentration in the rat brain were antagonized by desipramine (10 mg/kg i.p.).

6 It is suggested that DSP 4 primarily attacks the membranal noradrenaline uptake sites forming a covalent bond and that the nerve terminals, as a result of this binding, degenerate.

## Introduction

In previous papers (Ross, Johansson, Lindborg & Dahlbom, 1973; Ross & Renyi, 1976) it was reported that the alkylating compound *N*-2-chloroethyl-*N*-ethyl-2-bromobenzylamine hydrochloride (DSP 4) has a very sustained inhibitory action on the uptake of noradrenaline in mouse brain slices. The action was specific to the noradrenergic neurones, since no effect was obtained on the uptake of dopamine or 5-hydroxytryptamine (5-HT). DSP 4 was also found to be an active inhibitor of the noradrenaline uptake *in vitro*. One possible explanation of the effect is that the compound is covalently bound to the uptake sites *via* the reactive aziridinium derivative. However, the long-term effect can also be due to damage of the noradrenergic neurones resulting in their degeneration. In the present study the action of DSP 4 on the noradrenergic neurones was examined by determining the long-term effects on the concentration of noradrenaline, the enzyme dopamine-β-hydroxylase (DBH) activity and the noradrenaline accumulation in rat brain and heart. If the long-term effect is due to degeneration of the neurones all activities located in the noradrenaline neurones should decrease simultaneously.

## Methods

Male Sprague-Dawley rats weighing 150–180 g at the time of the injection of DSP 4 were used. The injections were given intraperitoneally and DSP 4 was dissolved in water immediately before use.

Uptake of [$^3$H]-(–)-noradrenaline, [$^3$H]-dopamine and [$^{14}$C]-5-HT in homogenates of various regions of the brain was determined according to Snyder & Coyle (1969) modified to the double labelling technique (Ross & Renyi, 1975). The brains were homogenized in 0.25 M sucrose (1:20 w/v) with an all-glass Potter-Elvehjem homogenizer. The homogenates were not centrifuged before use when the *in vivo* activity of DSP 4 was determined. The incubation was performed at 37°C in PVC centrifuge tubes for 5 min if not stated otherwise. The incubation mixture consisted of 100 µl of the homogenate, 0.2 nmol [$^{3}$H]-noradrenaline or [$^{3}$H]-dopamine, 0.2 nmol [$^{14}$C]-5-HT, 11 µmol glucose, 0.48 µmol pargyline, 2.2 µmol ascorbic acid and 0.26 µmol ethylenediaminetetra-acetic acid disodium salt (EDTA) in 1.8 ml of Krebs-Henseleit buffer, pH 7.4 (modified according to Snyder & Coyle, 1969). Immediately after incubation the tubes were chilled on an ice bath and centrifuged at 20,000 *g* for 20 min at 0°C. The pellets and the tubes were washed thrice with 5 ml of cold 0.9% w/v NaCl-solution (saline). The pellet was dissolved in 1.0 ml of Soluene-350 (Packard) at room temperature; 10 ml of the scintillation liquid (Permablend III, Packard) was added and the radioactivity was measured in two separate channels in a liquid scintillation spectrometer as described previously (Ross, Renyi & Ögren, 1972).

Dopamine-$\beta$-hydroxylase activity in brain homogenates was determined according to Coyle & Axelrod (1972) and that in heart homogenates according to Molinoff, Weinshilboum & Axelrod (1971) with the modification that the pH of the DBH assay was 5.5. Tyramine, 1 mM, was used as the substrate. The optimal $CuSO_4$ concentration was determined for each tissue and brain region.

Noradrenaline and dopamine in the rat brain were analyzed according to Chang (1964). 5-HT was determined according to Bogdanski, Pletscher, Brodie & Udenfriend (1956).

### *Drugs*

DSP 4 was synthesized by Dr Richard Dahlbom and collaborators, University of Uppsala, Sweden. Desipramine was a gift of Geigy AG and pargyline of Abbott Lab. Inc. (−)-Noradrenaline-[7-$^{3}$H-(N)] (3.8 Ci/mmol), dopamine-[$^{3}$H(G) (2.0 Ci/mmol) and *S*-adenosyl-L-methionine-[$^{14}$C-$CH_3$] (52 mCi/mmol) were purchased from NEN Chemicals GmbH, Frankfurt/Main, Germany and 5-hydroxytryptamine [2-side chain-$^{14}$C] creatinine sulphate (54 mCi/mmol) from The Radiochemical Centre, Amersham.

## Results

### *Inhibition of noradrenaline uptake*

The accumulation of [$^{3}$H]-(−)-noradrenaline in the synaptosomes of the homogenates of various regions of the brain was greatly decreased for a long time by a single intraperitoneal injection of DSP 4 50 mg/kg (Table 1); an exception was the uptake of noradrenaline by homogenates of the striatum which was not inhibited by DSP 4. This region is rich in dopaminergic neurones and the conclusion is that DSP 4 does not inhibit the uptake neurones in these neurones. The uptake of 5-HT was also unaffected by DSP 4 (Table 1). The small inhibition of the 5-HT uptake in the hypothalamus may be due to uptake of 5-HT into noradrenergic neurones. The selectivity of DSP 4 on noradrenaline uptake is in accordance with its *in vitro* activity. The $IC_{50}$ value for the inhibition of the noradrenaline uptake in homogenates of cerebral cortex was 2 µM whereas the corresponding value for the inhibition of the 5-HT uptake in the same homogenate was >30 µM (20% inhibition at this concentration). The dopamine uptake in a striatal homogenate was also only partially inhibited ($IC_{50} > 30$ µM, actually 40% inhibition at this concentration). Thus the affinity of DSP 4 for the noradrenaline uptake sites was more than ten times higher than for the 5-HT and dopamine uptake sites.

**Table 1** Effect of DSP 4 (50 mg/kg i.p.) on the accumulation of [$^{3}$H]-(−)-noradrenaline ([$^{3}$H]-NA) and [$^{14}$C]-5-hydroxytryptamine ([$^{14}$C]-5-HT) in homogenates of various regions of the rat brain one week after the injection

| | *Amine uptake as % in controls*[a] | |
|---|---|---|
| *Brain region* | *[$^{3}$H]-(−)NA* | *[$^{14}$C]-5-HT* |
| Frontal cerebral cortex[b] | 40 ± 5* | 102 ± 3 |
| Hypothalamus[c] | 50 ± 6* | 87 ± 3 |
| Striatum[d] | 100 ± 2 | 98 ± 6 |

Each value is the mean ± s.e. mean for 5 rats.

[a] Brain tissue was homogenized in 20 vol of 0.25 M sucrose. The incubation mixture consisted of 100 µl of the homogenate, 0.2 nmol [$^{3}$H]-(−)NA, 0.2 nmol [$^{14}$C]-5-HT in a final volume of 2.0 ml of Krebs buffer, pH 7.4. The incubation time was 10 min at 37°C. [b] Control uptake (nmol/g tissue (wet weight) in 10 min): [$^{3}$H]-NA: 0.34 ± 0.01; [$^{14}$C]-5-HT: 0.68 ± 0.05. [c] Control uptake: [$^{3}$H]-NA: 0.48 ± 0.04; [$^{14}$C]-5-HT 0.68 ± 0.05. [d] Control uptake: [$^{3}$H]-NA: 4.80 ± 0.22; [$^{14}$C]-5-HT: 1.98 ± 0.04.

*$P < 0.01$ (Student's *t*-test).

The dose-response of the inhibition of the noradrenaline in homogenates of frontal cerebral cortex one week after the intraperitoneal injection of DSP 4 gave an $ED_{50}$ value of approximately 20 mg/kg (Table 2).

In order to examine whether DSP 4 acutely inhibited the membranal uptake of noradrenaline, rats were treated with reserpine (5 mg/kg i.p.) one day before DSP 4, 50 mg/kg i.p., and the animals were killed 2 h after the injection of DSP 4. Although the uptake of the noradrenaline in the homogenates from the reserpine-treated rats was significantly lower than that in the control animals, DSP 4 caused an additional decrease, demonstrating that it did not act on the same sites as reserpine (Table 3). Further support for the hypothesis that DSP 4 acts primarily on the membranal transfer sites was obtained in experiments in which rats were pre-treated with desipramine (10 mg/kg i.p.) 15 min before the injection of DSP 4. As shown in Table 4 this pre-treatment antagonized the long-term effect of DSP 4 (50 mg/kg i.p.).

The noradrenaline accumulation in homogenates of frontal cerebral cortex and hypothalamus was compared at various times after the injection of DSP 4 (Figure 1). There was no significant difference in the inhibition in these two regions during the first weeks after the injection. However, the uptake of noradrenaline in the cerebral cortex was severely impaired at 14 weeks (59% inhibition, $P < 0.01$) and at 8 months (31%, $P < 0.05$) after a single injection of DSP 4 (50 mg/kg i.p.) but the noradrenaline uptake in hypothalamus was not reduced 14 weeks after the injection although there was still a significant inhibition (29%, $P < 0.05$) after 8 weeks.

The very long inhibitory effect of DSP 4 contrasted with its considerably shorter action on the uptake in the rat heart (Figure 1). The inhibition of the uptake of

**Table 2** Dose-response of the long-term effect of DSP 4 on the uptake of [$^3$H]-noradrenaline and dopamine-$\beta$-hydroxylase (DBH) activity in homogenate of the frontal cerebral cortex and the noradrenaline concentration in the whole rat brain

| *Dose* | *[$^3$H]-Noradrenaline uptake*[a] *(% of control)* | *DBH activity*[b] *(% of control)* | *Noradrenaline concentration*[c] *(% of control)* |
|---|---|---|---|
| 12.5 | 76±8* | 96±11 | 95±5 |
| 25 | 40±10** | 88±15 | 58±3** |
| 50 | 29±10** | 8±1** | 30±3** |

The rats were killed one week after the injection of DSP 4 (50 mg/kg i.p.). Each value is the mean ± s.e. mean from 5 rats.

*$P < 0.05$; **$P < 0.001$.

[a] Control uptake: 0.24±0.01 nmol/g in 5 min; [b] Control activity: 171±10 nmol $g^{-1}$ $g/h^{-1}$; [c] Control concentration: 0.476±0.024 µg/g.

**Table 3** Effect of reserpine on the inhibitory effect of DSP 4 on the noradrenaline uptake by cerebral cortical homogenate of the rat brain

| | *[$^3$H]-Noradrenaline uptake (nmol/g in 5 min)* | *% Inhibition* | P* |
|---|---|---|---|
| 1. Control | 0.29±0.02 | | |
| 2. DSP 4 | 0.11±0.04 | 62 | <0.01<br>(1–2) |
| 3. Reserpine | 0.17±0.01 | | <0.001<br>(1–3) |
| 4. Reserpine+ DSP 4 | 0.09±0.01 | 47 | <0.01<br>(3–4) |

Reserpine (5 mg/kg i.p.) was injected 16 h before DSP 4 (50 mg/kg i.p.). The rats were killed 2 h after DSP 4. Frontal cortex was homogenized in 20 vol of 0.25 M sucrose and 100 µl of the homogenate was taken for the assay. The incubation time was 5 minutes. The concentration of [$^3$H]-(—)-noradrenaline was 0.1 µM. Each value is the mean ± s.e. mean for 5 rats.

* Student's *t*-test.

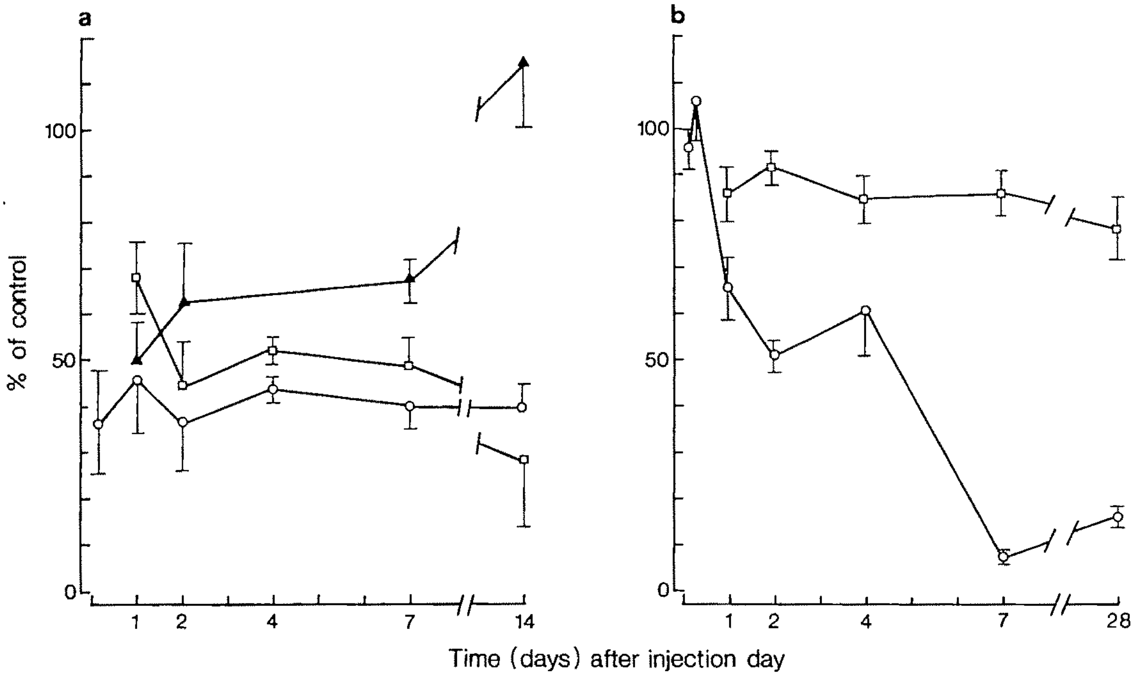


**Figure 1** Effects of DSP 4 (50 mg/kg i.p.) on (a) accumulation of [$^3$H]-noradrenaline (NA) in homogenates of frontal cerebral cortex (○), hypothalamus (□) and in heart atrium (▲) and (b) dopamine-β-hydroxylase (DBH) activity in homogenates of frontal cerebral cortex (○) and hypothalamus (□) of the rat at various time after the injection. Each value is the mean ± s.e. mean (vertical lines) for at least 5 rats and is expressed as a percentage of the control values, which were: [$^3$H]-NA uptake in cerebral cortex 0.28 ± 0.02 ($n$ = 28) nmol/g in 5 min; in hypothalamus: 0.38 ± 0.02 ($n$ = 24) nmol/g in 5 min; in atrium (cocaine-sensitive part): 0.181 ± 0.010 ($n$ = 16) nmol/g in 10 minutes. DBH activity in cerebral cortex (0.14 mM $CuSO_4$): 128 ± 4 ($n$ = 6) nmol octopamine $g^{-1}$ $h^{-1}$ and in hypothalamus (0.11 mM $CuSO_4$): 340 ± 15 ($n$ = 6) nmol $g^{-1}$ $h^{-1}$.

**Table 4** Antagonistic effect of desipramine (10 mg/kg i.p.) on the long-term action of DSP 4 (50 mg/kg i.p.) on the accumulation of [$^3$H]-noradrenaline (NA) in homogenates of frontal cerebral cortex, on the dopamine-β-hydroxylase (DBH) activity in the rat brain and heart and the noradrenaline concentration in the whole rat brain

| *Treatment* | *[$^3$H]-NA uptake*[a] *(% of control)* | *DBH activity* (% of control) *Brain*[b] | Heart[c] | *Noradrenaline in brain*[d] *(% of control)* |
|---|---|---|---|---|
| DSP 4 | 29 ± 10<br>($n$ = 6) | 42 ± 5<br>($n$ = 7) | 74 ± 5<br>($n$ = 5) | 49 ± 7<br>($n$ = 5) |
| Desipramine<br>+<br>DSP 4 | 68 ± 11*<br>($n$ = 5) | 102 ± 7**<br>($n$ = 6) | 95 ± 6*<br>($n$ = 5) | 77 ± 7*<br>($n$ = 5) |

Desipramine was injected 15 min before DSP 4. The rats were killed one week later. Each value is the mean ± s.e. mean expressed as a percentage of the control values. The number of animals are given in brackets.
[a] Control uptake: 0.44 ± 0.03 nmol/g in 10 min; [b] Control activity: 150 ± 7 nmol octopamine formed $g^{-1}$ $h^{-1}$;
[c] Control activity: 202 ± 10 nmol octopamine formed $g^{-1}$ $h^{-1}$; [d] Control level: 0.426 ± 0.025 μg/g.
*$P < 0.05$; **$P < 0.001$ (Student's $t$-test).

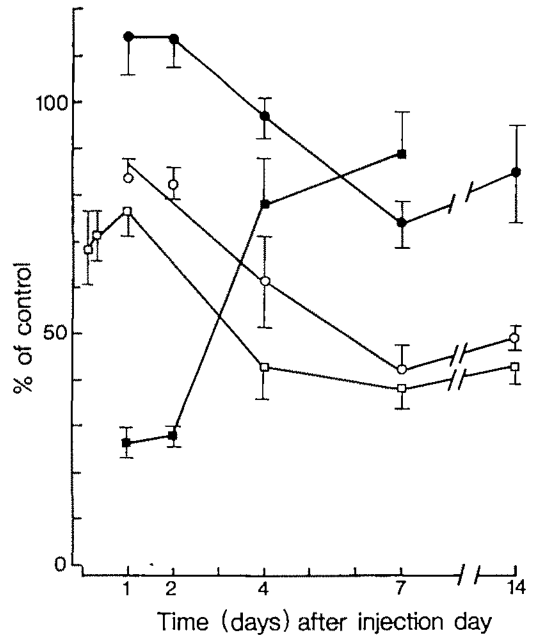


**Figure 2** Effects of DSP 4 (50 mg/kg i.p.) on the noradrenaline concentrations (squares) and the dopamine-β-hydroxylase (DBH) activity (circles) in whole brain (open symbols) and heart (solid symbols) of the rat at various time after injection. Each value is the mean ± s.e. mean (vertical lines) for 6 rats and is expressed as a percentage of the control values, which were: noradrenaline concentration in brain: 0.392 ± 0.031 μg/g; in heart: 0.716 ± 0.045 μg/g. DBH activity in brain: 145 ± 4 nmol $g^{-1}$ $h^{-1}$ and in heart 185 ± 11 nmol $g^{-1}$ $h^{-1}$.

noradrenaline in the rat atrium *in vitro* lasted only between one and two weeks after the injection of DSP 4.

*Dopamine-β-hydroxylase*

The DBH activity of brain homogenates was decreased by DSP 4 but in contrast to the inhibition of the noradrenaline uptake there was a slow onset of the decrease in DBH (Figure 2). In the heart there was a lag period of a few days before the DBH activity started to decrease, which then followed at the same rate as that in the brain. The DBH activity in heart did not decrease as much as in brain and recovered much earlier. DSP 4 added to brain homogenate did not inhibit the DBH activity at the concentration 3 μM.

The DBH activities in frontal cerebral cortex and hypothalamus were compared at various times after the injection of DSP 4 (Figure 1). The enzyme activity in the cortex was almost completely abolished after one week and stayed low for a long time. Thus, it was significantly ($P < 0.05$) lowered 8 months after the intraperitoneal injection of 50 mg/kg DSP 4 (control: 228 ± 16 nmol $g^{-1}$ $h^{-1}$; $n = 5$; DSP 4: 131 ± 28 nmol $g^{-1}$ $h^{-1}$; $n = 5$). The DBH activity in the hypothalamus was, on the other hand, much less affected. The lack of effect of DSP 4, 1 and 4 h after the injection, on the DBH activity indicates that DSP 4 in itself did not inhibit the enzyme. The dose-response curve (Table 2) showed that doses of DSP 4 lower than 50 mg/kg did not produce any long-term decrease in the DBH activity in cerebral cortex.

Since it has been shown that the DBH activity is regulated by a feed-back mechanism *via* the postsynaptic receptors (Molinoff, Brimijoin, Weinshilboum & Axelrod, 1970) it was of interest to examine whether inhibition of the membranal re-uptake of noradrenaline for a period of one week produced any effect on the brain DBH activity. Rats were therefore daily injected with desipramine (20 mg/kg i.p.) for one week and the DBH activity in the whole brain was determined on the eighth day. No difference between the treated and the control animals was obtained (DBH activity 134 ± 2 and 138 ± 3 nmol $g^{-1}$ $h^{-1}$, respectively).

Although the inhibition of the adrenaline uptake did not *per se* cause the fall in DBH activity it is possible that the binding of DSP 4 to the neuronal membrane produced such damage that a degenerative process was started. This hypothesis is supported by the observation that desipramine (10 mg/kg i.p.), which antagonized the inhibition of the noradrenaline uptake, completely prevented the decrease in brain and heart DBH activity (Table 4).

*Noradrenaline in brain and heart*

The noradrenaline concentration in the whole rat brain was decreased by DSP 4 (50 mg/kg i.p.) in a biphasic way (Figure 2). After an initial fall of approximately 30% a second fall in the noradrenaline concentration occurred one to 4 days after the injection whereafter it stayed approximately constant at 40% of the control level for at least two weeks. Desipramine (10 mg/kg i.p.) significantly antagonized the long-term decrease in noradrenaline produced by DSP 4 (Table 4).

A dose-response study performed one week after the injection of DSP 4 showed that 12.5 mg/kg was without any effect whereas 25 mg/kg had about half of the effect of 50 mg/kg on the brain noradrenaline (Table 2). Determination of noradrenaline in the cerebral cortex and hypothalamus one week after 50 mg/kg of DSP 4 showed that a marked decrease had occurred in both regions (Table 5).

The noradrenaline in the heart was differently affected compared with that in the brain (Figure 2) in that it rapidly decreased during the first two days but had recovered almost to normal after 4 days.

**Table 5** Effect of DSP 4 (50 mg/kg i.p.) on the noradrenaline concentration in cerebral cortex and hypothalamus one week after the injection

| | *Noradrenaline (μg/g)*<br>*Control* | <br>*DSP 4* | *% of control* |
|---|---|---|---|
| Cerebral cortex | 0.133 ± 0.004<br>($n=6$) | 0.046 ± 0.006**<br>($n=5$) | 35 |
| Hypothalamus | 0.608 ± 0.071<br>($n=5$) | 0.272 ± 0.049*<br>($n=5$) | 45 |

Each value is the mean ± s.e. mean of the number rats given in the brackets.
*$P<0.01$; **$P<0.001$ (Student's $t$-test).

### *Dopamine and 5-hydroxytryptamine in brain*

Dopamine in whole rat brain was not changed by DSP 4 (50 mg/kg i.p.) when examined at various times during the first week after the injection (Table 6). On the other hand the 5-HT concentration was significantly reduced by about 25% when determined one week after the injection (Table 6).

## Discussion

The long-term effect of DSP 4 on the accumulation of noradrenaline in synaptosomes of homogenates of the rat brain is in accordance with the previous observation of a sustained decrease in the uptake of noradrenaline in mouse brain slices (Ross *et al.*, 1973; Ross & Renyi, 1976). Since this effect was selective, rapid in onset, antagonized by desipramine and was independent of a functional intraneuronal storage mechanism, i.e. not sensitive to reserpine, it is likely that the primary action of DSP 4 is a selective inhibition of the membranal noradrenaline carrier sites. DSP 4 is an alkylating compound and may accordingly be covalently bound to these sites. The considerably shorter effect of DSP 4 on the noradrenaline uptake in heart atrium indicates a difference in the stability of this binding. The reaction sequence of the hypothelial binding may be as follows: DSP 4 having selective affinity for the noradrenaline transfer sites is bound by electrostatic attraction to the uptake sites and is there transformed to the aziridinium derivative, which reacts covalently with an anionic component of the site, the latter probably being identical with that group attracting the cationic amino group of noradrenaline. The stability of this covalent bond is dependent on the nature of this group, which can be a carboxylate, phosphate or sulphhydryl group. The difference in the sensitivity for DSP 4 between central and peripheral noradrenergic nerve terminals may be due to different stabilities of this bond. DSP 4 is rather rapidly cyclized to the aziridinium derivative under *in vitro* conditions ($T_{\frac{1}{2}}=7$ min at pH 7.4 (Ross *et al.*, 1973)), which means that the cyclization terminates within a rather short time after the injection.

**Table 6** Effect of DSP 4 (50 mg/kg i.p.) on the dopamine and 5-hydroxytryptamine (5-HT) concentrations in the whole rat brain.

| *Time after injection (days)* | *Dopamine*[a]<br>*(% of control)* | *5-HT*[b] |
|---|---|---|
| 1 | 106 ± 4 | 88 ± 4* |
| 2 | — | 84 ± 5* |
| 4 | 97 ± 3 | 78 ± 5* |
| 7 | 115 ± 11 | 77 ± 6* |

Each value is the mean ± s.e. mean from at least 5 rats.
*$P<0.05$ (Student's $t$-test).
[a] Control value: 0.543 ± 0.038 μg/g($n=5$); [b] control value: 0.734 ± 0.021 μg/g ($n=10$).

The decrease in DBH activity in the rat brain and heart indicates another effect of DSP 4. Since this effect had a much slower onset than the decrease in uptake and was antagonized by desipramine it seems to be secondary to the uptake inhibition. The observation that higher doses of DSP 4 had to be given in order to affect DBH activity than are needed to affect noradrenaline uptake also supports this hypothesis. The finding that DBH in cerebral cortex was much more sensitive to DSP 4 than was that in the hypothalamus is interesting, since it indicates that the noradrenergic nerve terminals in the cortex are much more sensitive to this effect of DSP 4 than are the noradrenergic axons and cell bodies in the hypothalamus. This regional difference was also observed for the long-term inhibitory effect of DSP 4 on the noradrenaline uptake but because of the sustained inhibition of the uptake mechanism this difference could not be demonstrated until after two months. These results suggest that a degenerative process is taking place in the nerve terminals. Such degeneration will of course also affect other constituents of the noradrenergic nerves, e.g. noradrenaline itself.

The finding of a long-term decrease in the noradrenaline content of the rat brain after DSP 4 gives further support to the suggestion that a degeneration of noradrenergic nerve terminals takes place. However, the effect on noradrenaline differed from that on DBH in that the hypothalamus was affected almost as much as the cerebral cortex. One possible explanation of this discrepancy may be that whole cerebral cortex was used for the determination of noradrenaline whereas about 40 mg of the frontal cerebral cortex was used for the assay of DBH activity. The significant antagonism by desipramine of the reduction in the noradrenaline concentration is in accordance with the hypothesis that the long-term decrease in noradrenaline is a secondary phenomenon and that the primary attack is in alkylation of the membranal uptake sites.

Since the 25% fall in the 5-HT concentration induced by DSP 4 in the whole rat brain did not correspond to a similar decrease in the 5-HT uptake this effect of DSP 4 does not appear to be due to a degeneration of 5-HT neurones. The origin of this effect has to be examined in further experiments.

Because of its long-term selective degeneration of the noradrenergic nerve terminals DSP 4 may become a valuable tool in studies of the functional actions of noradrenline in the brain. Compared with 6-hydroxydopamine, the compound now used for this purpose, DSP 4 has the advantage of readily passing the blood-brain barrier and can accordingly be injected parenterally. Furthermore, it seems to be more selective than 6-hydroxydopamine, which also affects dopamine neurones (Ungerstedt, 1968). However, before DSP 4 can be used as a pharmacological tool its action on central and peripheral monoaminergic receptor mechanisms has to be evaluated. Such studies are now being performed.

I thank Mrs Öie Frödén and Miss Diana Kelder for skilful technical assistance and Professor Richard Dahlbom and Dr Björn Lindborg for the synthesis of DSP 4.

## References

BOGDANSKI, D.F., PLETSCHER, A., BRODIE, B.B. & UDENFRIEND, S. (1956). Identification and assay of serotonin in brain. *J. Pharm. exp. Ther.*, **117**, 82–88.

CHANG, C.C. (1964). A sensitive method for spectrophotofluorometric assay of catecholamines. *Int. J. Neuropharmac.*, **3**, 643–649.

COYLE, J.T. & AXELROD, J. (1972). Dopamine-$\beta$-hydroxylase in the rat brain: developmental characteristics. *J. Neurochem.*, **19**, 449–459.

MOLINOFF, P.B., BRIMIJOIN, S., WEINSHILBOUM, R. & AXELROD, J. (1970). Neurally mediated increase in dopamine-$\beta$-hydroxylase activity. *Proc. Nat. Acad. Sci., USA*, **66**, 453–458.

MOLINOFF, P.B., WEINSHILBOUM, R. & AXELROD, J. (1971). A sensitive enzymatic assay for dopamine-$\beta$-hydroxylase. *J. Pharmac. exp. Ther.*, **178**, 425–431.

ROSS, S.B., JOHANSSON, J.G., LINDBORG, B. & DAHLBOM, R. (1973). Cyclizing compounds. I. Tertiary *N*-(2-bromobenzyl)-*N*-haloalkylamine with adrenergic blocking action. *Acta pharmaceutica Suecica*, **10**, 29–42.

ROSS, S.B. & RENYI, A.L. (1975). Tricyclic antidepressant agents. I. Comparison of the inhibition of the uptake of $^3$H-noradrenaline and $^{14}$C-5-hydroxytryptamine in slices and crude synaptosome brain. *Acta pharmac. tox.*, **36**, 382–394.

ROSS, S.B. & RENYI, A.L. (1976). On the long-lasting inhibitory effect on *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP 4) on the active uptake of adrenaline. *J. Pharm. Pharmac.*, **28**, 458–459.

ROSS, S.B., RENYI, A.L. & ÖGREN, S.-O. (1972). Inhibition of the uptake of noradrenaline and 5-hydroxytryptamine by chlorphentermine and chlorimipramine. *Eur. J. Pharmac.*, **17**, 107–112.

SNYDER, S.H. & COYLE, J.T. (1969). Regional difference in $^3$H-norepinephrine and $^3$H-dopamine uptake into rat brain homogenates. *J. Pharmac. exp. Ther.*, **165**, 78–86.

UNGERSTEDT, U. (1968). 6-Hydroxydopamine induced degeneration of central monoamine neurones. *Eur. J. Pharmac.*, **5**, 107–110.

*(Received March 15, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 529–531

# INHIBITION BY AMILORIDE OF SODIUM-DEPENDENT FLUID REABSORPTION IN THE RAT ISOLATED CAUDAL EPIDIDYMIS

P.Y.D. WONG & C.H. YEUNG

Department of Physiology, Medical Faculty, University of Hong Kong, Hong Kong

1 The rate of fluid reabsorption was studied in the rat isolated caudal epididymal sac *in vitro*.

2 Part of the fluid reabsorption was found to be dependent on intraluminal $Na^+$. Amiloride (0.1 mM) completely inhibited this component of fluid reabsorption.

3 The log dose-inhibition curve to amiloride was sigmoid and the $IC_{50}$ value was found to be 1.6 μM.

## Introduction

Amiloride is a potent inhibitor of passive $Na^+$ transport in many tissues (see Cuthbert, 1974). For example, in mammalian tissues, it inhibits passive sodium transfer in the distal tubule of the rat kidney (Guignard & Peters, 1970) and in the salivary duct of rat (Schneyer, 1970). In this paper we report an inhibitory effect of amiloride on the $Na^+$-dependent fluid reabsorption in the rat caudal epididymis.

## Methods

Fluid reabsorption was studied in the isolated caudal epididymis of rats *in vitro*. Male rats weighing between 200–300 g were killed by a blow on the head. The epididymis was quickly removed and placed in cold Krebs bicarbonate solution. A segment of the caudal epididymis (about 1 cm long) was dissected free of fat and connective tissue and placed on a specially designed platform similar to the one used for measuring secretory rate in isolated seminiferous tubules (Cheung, Hwang & Wong, 1976). Each end was clamped on to the platform, and an incision made in each end of the segment close to the clamps. The lumen was then flushed with Krebs bicarbonate solution to remove all the spermatozoa. One end of the segment was closed with a silk ligature, and the tubule filled with about 0.25 μl Krebs bicarbonate solution by means of polyethylene cannula with tip diameter of about 80 μm, after which the other end of the segment was also closed. The whole operation was performed under a dissecting microscope (16 × magnification). The small sac of epididymis (about 2 mm long), supported on the platform, was then placed in a small bath (volume 2 ml)) filled with normal Krebs bicarbonate solution which was bubbled continuously with 5% $CO_2$ in $O_2$. The bathing solution was maintained at 35°C, the temperature being monitored by a thermistor probe.

To estimate the rate of fluid reabsorption, the bath was placed under a microscope (Olympus, 40 × magnification). The internal diameter of the lumen was measured at intervals of 0.4 mm along the length of the sac, using an eyepiece micrometer. The mean luminal diameter of the epididymis was obtained and by knowing the length of the sac, the luminal volume was calculated. Readings of the diameters were taken at 10 min intervals. A reduction in the luminal volume indicated a net reabsorption of fluid in the duct. The rate of fluid reabsorption was expressed in μl of fluid reabsorbed per $cm^2$ of the tubule per 30 minutes. The reabsorptive area was calculated from the mean radius of the tubule and its length ($2\pi rl$). The peritubular and intraluminal fluids were always iso-osmotic.

The Krebs bicarbonate solution used had the following composition: (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.56, $MgSO_4$ 1.13, $NaH_2PO_4$ 1.17, $NaHCO_3$ 25 and glucose 11.1. When gassed with 5% $CO_2$ in $O_2$, it had a pH of 7.4. When sodium-free solution was used the NaCl was substituted by an equivalent amount of choline chloride and the $NaHCO_3$ by $KHCO_3$ (5.9 mM). In this instance the solution was bubbled with pure $O_2$ to obtain a pH of 7.4. The amiloride was added to the intraluminal fluid only.

## Results

The rate of fluid reabsorption under various conditions is shown in Figure 1a. The basal rate was found to be $2.63 \pm 0.22$ μl $cm^{-2}$ 30 $min^{-1}$ (mean ± s.e., $n = 11$).

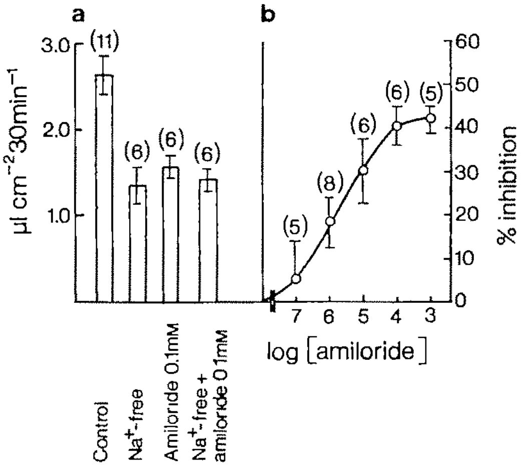


**Figure 1(a)** Rate of fluid reabsorption by rat isolated caudal epididymal sac *in vitro*. Each column represents the mean ± s.e. The number of experiments is shown in parentheses. (b) Log concentration—% inhibition curve for amiloride on fluid reabsorption in the isolated caudal epididymal sac. Each point shows the mean ± s.e. with the number of observations shown in parentheses.

When the lumen was filled with sodium-free solution, the rate of fluid reabsorption was reduced to $1.35 \pm 0.22$ μl cm$^{-2}$ 30 min$^{-1}$ mean ± s.e., $n=6$). These values are significantly different ($P < 0.001$) (Student's *t* test). When amiloride (0.1 mM) was added to the intraluminal fluid, the rate of fluid reabsorption was $1.56 \pm 0.13$ μl cm$^{-2}$ 30 min$^{-1}$ (mean ± s.e., $n=6$). This value is not significantly different from that obtained when the lumen contained Na$^+$-free solution. However, the addition of amiloride (0.1 mM) to the sodium-free solution had no effect on the rate of fluid reabsorption, the value being $1.42 \pm 0.14$ (mean ± s.e., $n=6$). These results suggest that part of the fluid reabsorption in the isolated caudal epididymis is dependent on the presence of sodium ions in the luminal fluid; and that this component of fluid reabsorption can be blocked completely by amiloride. However, the component of fluid reabsorption that was independent of the presence of sodium ions appeared to be unaffected by amiloride.

The dose-response curve for the inhibition of epididymal fluid reabsorption by amiloride is shown in Figure 1b. The curve is sigmoid and the concentration of amiloride giving 50% inhibition was found to be 1.6 μM. Maximal inhibition was produced by 0.1 mM.

## Discussion

It is known that the composition of the fluid which surrounds the spermatozoa in the epididymis changes along the length of the duct and that consequently, the milieu surrounding the spermatozoa in the caudal epididymis is considerably different from that in the testis. This 'milieu' is intimately concerned with the development of the spermatozoa after they leave the testis. As the spermatozoa pass down the epididymis they encounter a decreasing Na$^+$/K$^+$ ratio as well as decreasing Na$^+$ and K$^+$ concentrations (Mann, 1974). In the rabbit, the caudal epididymal fluid contains very low concentrations of sodium and chloride, but these concentrations increase after castration (Jones & Glover, 1975). This effect can be reversed by treatment with a testosterone implant. Levine & Marsh (1971) have measured the transluminal potential in different parts of the rat epididymis *in vivo*; they found that, in the caudal region, the potential was 25 mV with the inside negative with respect to the outside. This potential difference might be caused by an active transport of sodium from the lumen into the peritubular fluid. As the spermatozoa passed down the epididymis, the spermatocrit increased reflecting a reabsorption of fluid from the lumen of the duct.

In our experiments on the isolated caudal epididymis, we have found that when the intraluminal and peritubular fluids are initially of identical composition, a net reabsorption of fluid occurs, as shown by a decrease in intraluminal volume. The rate of reabsorption is temperature-dependent and is inhibited by 2, 4 dinitrophenol suggesting that the reabsorption is an energy-dependent process (unpublished). The present results show that part of this fluid reabsorption is dependent on intraluminal sodium, as complete removal of intraluminal Na$^+$ caused a 48.7% reduction in fluid reabsorption. It is possible that this part of fluid reabsorption is secondary to an active transport of sodium from the intraluminal to the peritubular fluid. The mechanism for sodium transport may involve a passive diffusion of Na$^+$ ions across the luminal membrane and an active extrusion step across the peritubular membrane. The addition of amiloride to the intraluminal fluid caused a decrease in Na$^+$-dependent fluid reabsorption; this is consistent with an inhibition by amiloride of the passive Na$^+$ entry step at the luminal membrane. The IC$_{50}$ for amiloride on fluid reabsorption in the caudal epididymis was found to be 1.6 μM. This value is comparable to that causing 50% inhibition of passive Na$^+$ transport in the distal tubule of the kidney. (Guignard & Peters, 1970.)

The authors are grateful to Merck Sharp and Dohme for the supply of amiloride and to Dr A.W. Cuthbert for reading the manuscript. This work was supported by W.H.O. grant to P.Y.D.W.

## References

CHEUNG, Y.M., HWANG, J.C. & WONG, P.Y.D. (1976). *In vitro* measurement of the secretory rate in isolated seminiferous tubules of rats. *J. Physiol., Lond.,* **257**, 17P.

CUTHBERT, A.W. (1974). Characteristics of sodium channels in transporting epithelia. In *Drugs and transport processes*, ed. Callingham, B.A. pp. 173–184. London: Macmillan.

GUIGNARD, J.P. & PETERS, G. (1970). Effects of triameterene and amiloride on urinary acidification and potassium excretion in the rat. *Eur. J. Pharmac.,* **10**, 255–267.

JONES, R. & GLOVER, T.D. (1975). Interrelationships between spermatozoa, the epididymis and epididymal plasma. In *The Biology of the Male Gamete,* ed. Duckett, J.G. & Racey, P.A. Supplement No. 1 to the Biological Journal of the Linnean Society, Vol. 7, pp. 367–384.

LEVINE, N. & MARSH, D.J. (1971). Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. *J. Physiol., Lond.,* **213**, 557–570.

MANN, T. (1974). Biochemical aspects of sperm maturation. In *Male Fertility and Sterility,* ed. Mancini, R.E. & Martini, L. London: Academic Press.

SCHNEYER, L.H. (1970). Amiloride inhibition of ion transport in perfused excretory duct of rat submaximally gland. *Am. J. Physiol.,* **219**, 1050–1055.

*(Received March 17, 1976.*
*Revised June 3, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 533–545

# EFFECT OF AMINOPHYLLINE ON TRYPTOPHAN AND OTHER AROMATIC AMINO ACIDS IN PLASMA, BRAIN AND OTHER TISSUES AND ON BRAIN 5-HYDROXYTRYPTAMINE METABOLISM

G. CURZON & J.C.R. FERNANDO
Department of Neurochemistry, Institute of Neurology, Queen Square, London WC1N 3BG

1 Aminophylline and other methylxanthines increase brain tryptophan and hence 5-hydroxytryptamine turnover. The mechanism of this effect of aminophylline was investigated.

2 At lower doses (≯ 100 mg/kg i.p.) the brain tryptophan increase could be explained by the lipolytic action of the drug, i.e. increased plasma unesterified fatty acid freeing plasma tryptophan from protein binding so that it became available to the brain.

3 Plasma unesterified fatty acid did not increase when aminophylline (100 mg/kg i.p.) was given to nicotinamide-treated rats but as both plasma total and free tryptophan rose, a tryptophan increase in the brain still occurred.

4 The rise in brain tryptophan concentration following the injection of a higher dose of the drug (150 mg/kg i.p.) could no longer be explained by a rise of plasma free tryptophan as the ratio of brain tryptophan to plasma free tryptophan rose considerably. Plasma total tryptophan fell and the plasma insulin concentration rose.

5 The increase of brain tryptophan concentration after injection of 150 mg/kg aminophylline appeared specific for this amino acid as brain tyrosine and phenylalanine did not increase. However as their plasma concentrations fell the brain/plasma ratio for all three amino acids rose.

6 The higher dose of aminophylline increased the muscle concentration of tryptophan but that of tyrosine fell and that of phenylalanine remained unaltered. The liver concentrations were not affected.

7 The aminophylline-induced increases of the ratio of brain tryptophan to plasma free tryptophan no longer occurred when the drug was given to animals injected with the $\beta$-adrenoceptor blocking agent propranolol or the diabetogenic agent streptozotocin.

8 The changes in brain tryptophan upon aminophylline injection may be explained by (a) increased availability of plasma tryptophan to the brain due to increased lipolysis and (b) increased effectiveness of uptake of tryptophan by the brain due to increased insulin secretion.

## Introduction

Much attention has recently been paid to mechanisms by which brain tryptophan concentration is controlled. This is principally because tryptophan hydroxylase, the rate limiting enzyme for brain 5-hydroxytryptamine (5-HT) synthesis is normally unsaturated with its precursor tryptophan (Eccleston, Ashcroft & Crawford, 1965; Friedman, Kappelman & Kaufman, 1972) so that alterations of brain tryptophan concentration can lead to changes of brain 5-HT turnover.

There is evidence that the following three factors can influence brain tryptophan concentration:

(a) Changes in the concentration of the relatively small fraction of unbound tryptophan in plasma (Knott & Curzon, 1972; Tagliamonte, Biggio, Vargiu & Gessa, 1973). This fraction can be increased in conditions in which the plasma concentration of unesterified fatty acids (UFA) increases as these substances decrease the binding of tryptophan to albumin (Knott & Curzon, 1972; Curzon, Friedel & Knott, 1973; Curzon, Kantamaneni, Winch, Rojas-Bueno, Murray-Lyon & Williams, 1973; Curzon & Knott, 1974; Knott & Curzon, 1975).

(b) Competition between tryptophan and a group of neutral amino acids for uptake by the brain This has been shown *in vitro* (Kiely & Sourkes, 1972) and *in vivo* (Oldendorf, 1971). The tryptophan concentrations used in these experiments were considerably greater than those of free tryptophan in plasma. However, this mechanism could be involved in the control of brain tryptophan concentration following food intake as brain tryptophan then no longer correlates with free plasma tryptophan (Madras, Cohen, Messing, Munro & Wurtman, 1974).

It has also been used to explain increased brain tryptophan in liver failure by Munro, Fernstrom & Wurtman (1975) who suggested that plasma insulin elevation in this disorder leads to a fall in the plasma concentrations of branched chain amino acids competing with tryptophan for transport to the brain. Although brain tryptophan changes in various kinds of acute liver failure have been explained largely in terms of plasma free tryptophan changes (Curzon, Knott, Murray-Lyon, Record & Williams, 1975) insulin injection does lead to an increase of brain tryptophan or of the ratio of its concentration to that of plasma free tryptophan (Fernstrom & Wurtman, 1971; Fernando, Knott & Curzon, 1976).

(c) Effectiveness of tryptophan uptake by the brain. This exhibits diurnal variations and may be affected by the activity of central catecholaminergic systems (Hery, Rouer, Kan & Glowinski, 1974) and cyclic adenosine-3′,5′-monophosphate (cyclic AMP) (Tagliamonte, Tagliamonte, Forn, Perez-Cruet, Krishna & Gessa, 1971).

One group of drugs which increase brain 5-HT turnover are the methylxanthines, e.g. aminophylline (Berkowitz & Spector, 1971). The increased 5-HT turnover due to aminophylline is associated with an increase in brain tryptophan concentration (Curzon & Knott, 1974) which appears to be related to inhibition of cyclic AMP breakdown in fat cells (Butcher, Ho, Meng & Sutherland, 1975) leading to an elevation of UFA and hence of free tryptophan in the plasma. However, the catecholamine releasing (Wooten, Thoa, Kopin & Axelrod, 1973), insulin releasing (Turtle, Littleton & Kipnis, 1967) or other actions of aminophylline could also be involved.

The present paper describes an investigation of the roles of plasma free tryptophan and insulin in the action of aminophylline on brain concentrations of tryptophan and on brain 5-HT metabolism. The effect of aminophylline on the disposition of other aromatic amino acids was also studied.

## Methods

Male Sprague-Dawley rats (170–220 g, Anglia Laboratory Animals, Alconbury, Hunts.) were caged in groups of three or four and kept under a 06 h 00 min–18 h 00 min light-dark cycle in an acoustically lagged housing at 24 ± 1 °C and fed with Oxoid 41B pellets and water *ad libitum*. The animals were killed by guillotine between 14 h 30 min and 16 h 30 min and blood collected into heparin-containing tubes except when required for insulin assay when citrate was used. It was immediately centrifuged and the plasma stored at −20 °C. Separate samples were stored for insulin assay. The brains were removed rapidly, frozen with solid $CO_2$ and stored at −20 °C.

The following drugs were used: aminophylline injection B.P. (Evans Medical), nicotinamide, propranolol (ICI), streptozotocin (Upjohn, Kalamazoo, Mich.,) dissolved in 0.07 M pH 4.5 sodium citrate buffer, caffeine citrate (BDH) and theobromine (BDH). Drugs supplied as solids were dissolved in 0.9% w/v Na Cl solution (saline) unless otherwise stated. Injection volume was 2.5 ml/kg body weight. Control animals were injected with the appropriate vehicles.

### *Analytical methods*

Plasma total and ultrafilterable tryptophan, brain tryptophan, 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) and plasma and brain tyrosine were determined as previously described (Curzon, Joseph & Knott, 1972; Knott & Curzon, 1972). Phenylalanine was determined by the method of McCaman & Robins (1962) in plasma and in a 50 μl portion of the brain extract used for tyrosine determination (Curzon *et al.*, 1972). Tryptophan, tyrosine and phenylalanine were also determined in gastrocnemius muscle and liver by the above methods. Plasma UFA were determined fluorometrically (Curzon & Kantamaneni, unpublished) and plasma insulin by radioimmunoassay (Kit method, Lepetit, Maidenhead, Berks.).

## Results

### *Behavioural effects of aminophylline*

Rats given 50–150 mg/kg aminophylline intraperitoneally were more responsive than control ainmals to auditory, visual and tactile stimuli at about 45 min after injection. This soon disappeared and at 2 h the animals appeared sedated and flaccid and at 3 h were curled up and cool to the touch.

### *Effect of aminophylline on plasma unesterified fatty acids and tryptophan and on brain tryptophan and brain 5-hydroxytryptamine metabolism*

Figure 1 shows that during the 3 h period after an injection of aminophylline (150 mg/kg i.p.) the total tryptophan concentration in the plasma gradually fell. The changes were significant at 2 and 3 hours. Plasma UFA and the percentage of plasma tryptophan which was unbound (i.e. ultrafilterable) did not alter significantly until the third hour when both rose to about twice their initial values. However, as total tryptophan fell the absolute increase in free tryptophan was not significant. The brain concentrations of tryptophan and 5-HIAA were significantly increased 1, 2 and 3 h after aminophylline injection (Figure 2) but not that of 5-HT. The brain changes did not simply reflect plasma free tryptophan changes as the ratio: brain tryptophan/plasma free tryptophan rose (significantly at 2 hours).

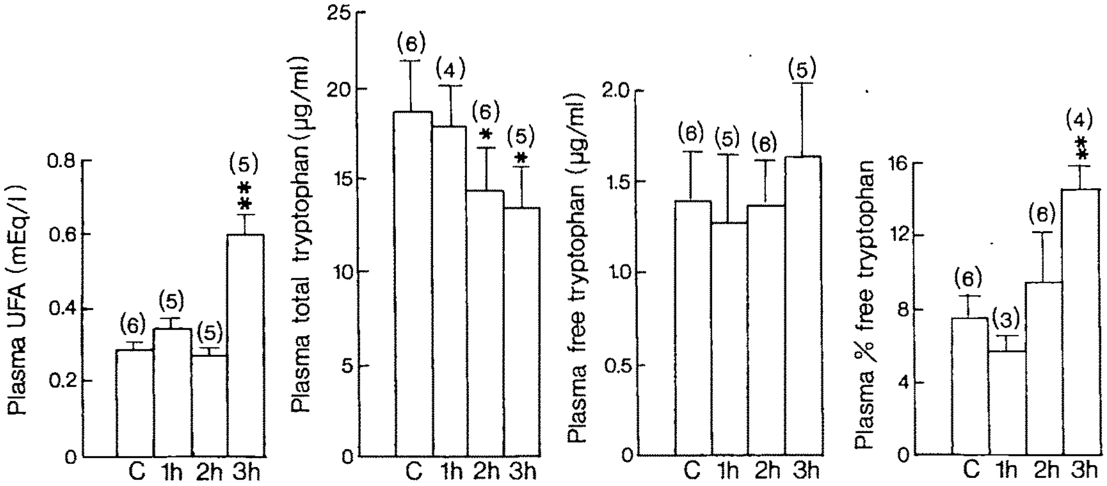


**Figure 1** Time course of effect of aminophylline (150 mg/kg, i.p.) on the plasma concentrations of unesterified fatty acids (UFA) and tryptophan. Rats were killed 1, 2 or 3 h after the drug injection. Control animals were injected with saline. Control animals killed 1, 2 or 3 h after the injection did not show significantly different biochemical values and combined results are shown in column C. Results are expressed as means + one s.d. Numbers of determinations shown in parentheses. Significance of differences from controls (Student's *t* test): $^*P<0.02$; $^{**}P<0.001$.

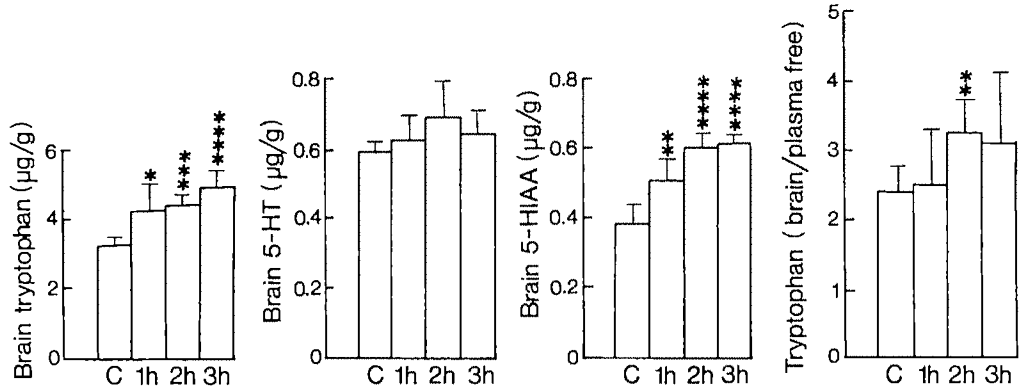


**Figure 2** Time course of effect of aminophylline (150 mg/kg, i.p.) on brain indoles. Treatments as described under Figure 1. Results are expressed as means + one s.d. using 5–6 rats per group. Significance of differences from controls (Student's *t* test): $^*P<0.05$; $^{**}P<0.02$; $^{***}P<0.01$; $^{****}P<0.001$.

Table 1 shows the effects of 50, 100 and 150 mg/kg of aminophylline 3 h after intraperitoneal injection. Plasma total tryptophan fell significantly at the 150 mg/kg dose (as shown in Figure 1) At the lower dose it only altered slightly. UFA rose significantly at all doses but without apparent dose-dependence. There were associated rises in the percentage of free tryptophan in the plasma (not shown) and at the two lower doses the free tryptophan concentration in the plasma rose considerably and significantly. However, presumably because of the associated fall of total plasma tryptophan the concentration of free tryptophan did not rise at the highest drug dose. Nevertheless brain tryptophan and 5-HIAA concentrations rose significantly at all doses. The percentage increases of brain tryptophan and 5-HIAA were only slightly higher as the dose of aminophylline increased and were comparable for both substances. As in earlier work (Curzon & Knott, 1974) 5-HT rose slightly but significantly at all doses. The ratios of brain tryptophan to plasma free tryptophan were not significantly altered by the lower two doses of

**Table 1** Effect of different doses of aminophylline on plasma unesterified fatty acid (UFA) and tryptophan metabolism

| | Plasma | | | Brain (µg/g) | | | Brain tryptophan / Plasma free |
|---|---|---|---|---|---|---|---|
| | UFA (mEq/l) | Tryptophan (µg/ml) | | Tryptophan | 5-HT | 5-HIAA | |
| *Injected* | | Total | Free | | | | |
| Saline | 0.30±0.13<br>(6) | 10.09±1.39<br>(6) | 1.03±0.06<br>(6) | 1.69±0.15<br>(5) | 0.33±0.03<br>(6) | 0.32±0.07<br>(6) | 1.62±0.11<br>(4) |
| Aminophylline 50 mg/kg | 0.64±0.23*<br>(6) | 11.10±0.98<br>(6) | 1.80±0.39**<br>(6) | 2.50±0.31***<br>(6) | 0.38±0.02**<br>(6) | 0.52±0.03***<br>(6) | 1.42±0.27<br>(6) |
| Saline | 0.42±0.23<br>(12) | 13.07±2.16<br>(13) | 1.87±0.45<br>(11) | 2.64±0.34<br>(13) | 0.50±0.04<br>(13) | 0.41±0.04<br>(13) | 1.46±0.33<br>(12) |
| Aminophylline 100 mg/kg | 0.84±0.28***<br>(13) | 15.26±2.47*<br>(13) | 2.51±0.53**<br>(13) | 4.16±0.92***<br>(13) | 0.57±0.05***<br>(13) | 0.67±0.09***<br>(13) | 1.74±0.62<br>(13) |
| Saline | 0.29±0.10<br>(6) | 14.15±1.71<br>(6) | 0.91±0.24<br>(6) | 2.57±0.10<br>(6) | 0.36±0.01<br>(6) | 0.31±0.03<br>(6) | 2.63±0.43<br>(5) |
| Aminophylline 150 mg/kg | 0.56±0.15***<br>(6) | 9.13±2.12**<br>(6) | 0.93±0.17<br>(6) | 4.55±0.56***<br>(6) | 0.47±0.02***<br>(6) | 0.55±0.06***<br>(6) | 4.27±0.19**<br>(4) |

Experiments at different doses were not performed concurrently. Injections were made intraperitoneally and rats killed 3 h later. Results on two separate groups of rats given 100 mg/kg aminophylline are combined.

Results are expressed as means ± one s.d. Nos. of determinations are shown in parentheses. Results compared by Student's *t* test; Significance of difference from saline controls: $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

aminophylline suggesting that the brain changes at these doses largely reflected those of the plasma concentrations of free tryptophan but at the 150 mg/kg dose this ratio was considerably increased.

### *Effect of aminophylline (100 mg/kg) on plasma unesterified fatty acids and tryptophan and brain tryptophan in nicotinamide-treated or fasted rats*

The relationship between the effects of aminophylline on plasma UFA and on tryptophan and its metabolism was studied by the injection of aminophylline at a dose of 100 mg/kg. The effect of this dose on brain tryptophan can be explained by the increase in the plasma concentration of free tryptophan due to increased plasma UFA (Table 1). It was given either with nicotinamide (250 mg/kg) which prevents lipolysis (Dalton, Van Trabert & Dwyer, 1970) or after depriving rats of food for 24 h to promote lipolysis. Results are shown in Table 2. In nicotinamide-treated rats aminophylline increased neither plasma UFA nor the percentage of free tryptophan (not shown) 3 h later. However, as plasma total tryptophan concentration increased significantly the absolute concentration of plasma free tryptophan also increased and brain values rose comparably.

As previously found (Knott & Curzon, 1972), fasting increased both plasma free tryptophan and brain tryptophan comparably. Treatment of fasted rats with aminophylline led to a fall of plasma total tryptophan concentration and to a further increase of plasma free tryptophan concentration so that in contrast with the normal and the nicotinamide experiment the percentage of plasma tryptophan in the free state rose considerably (even though an increase of plasma UFA was not demonstrable). Brain tryptophan concentration increased by 55% and plasma free tryptophan concentration by 25%. Comparison with results in Table 1 did not suggest that fasted and fed rats responded differently to aminophylline.

### *Effect of aminophylline (150 mg/kg) on plasma unesterified fatty acids and insulin and on plasma and brain aromatic amino acids*

The mechanism by which the higher dose of aminophylline increased brain tryptophan without apparently increasing the concentration of plasma free tryptophan (Table 1, Figures 1 and 2) was investigated further by studying the effects of aminophylline (150 mg/kg i.p.) not only on the concentrations of tryptophan but also on those of tyrosine and phenylalanine in plasma and brain 3 h after the injection in order to see whether the effect was specific to tryptophan. Plasma insulin was also determined to see whether a relation exists between the amino acid changes and the effect of aminophylline on insulin

**Table 2** Effect of aminophylline on plasma unesterified fatty acid (UFA) and tryptophan disposition in nicotinamide treated and food-deprived rats

| | Plasma | | | Brain tryptophan | Brain tryptophan |
|---|---|---|---|---|---|
| Treatment | UFA (mEq/l) | Tryptophan (µg/ml) Total | Free | (µg/g) | Plasma free tryptophan |
| Nicotinamide (250 mg/kg) | 0.32±0.33 (12) | 14.47±2.90 (13) | 1.84±0.51 (13) | 2.76±0.40 (13) | 1.56±0.26 |
| Nicotinamide (250 mg/kg) + aminophylline (100 mg/kg) | 0.24±0.13 (13) | 20.29±3.97*** (13) | 2.61±0.33*** (11) | 4.34±0.53*** (13) | 1.72±0.34 (11) |
| Fed+saline (6) | 0.53±0.29 | 16.18±2.71 | 2.27±0.20 | 2.94±0.25 | 1.30±0.15 |
| Fasted 24 h+ saline (6) | 1.09±0.37† | 15.83±2.69 | 2.97±0.37†† | 3.75±0.39†† | 1.28±0.23 |
| Fasted 24 h+ aminophylline (100 mg/kg) (5) | 0.99±0.22 | 11.71±1.23* | 3.71±0.20** | 5.80±0.53*** | 1.58±0.38 |

Injections were made intraperitoneally and rats killed 3 h later. Results on rats given nicotinamide are combined from two separate experiments.

Results are expressed as means ± one s.d. Nos. of determinations are shown in parentheses. Results compared by Student's $t$ test. Significance of differences from corresponding nicotinamide or fasted and saline-injected controls: * $P<0.02$; ** $P<0.01$; *** $P<0.001$. Significance of difference from fed controls: † $P<0.02$; †† $P<0.01$.

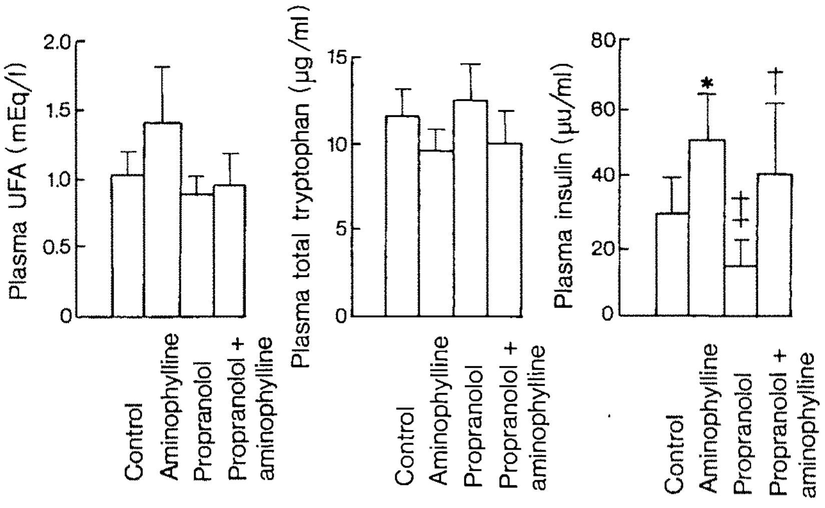


**Figure 3** Effect of aminophylline (150 mg/kg i.p.) and propranolol (2 mg/kg i.p., ×2) on plasma concentrations of unesterified fatty acids (UFA), total tryptophan and insulin. Aminophylline was injected 3 h before killing and propranolol injected 3 h and 1 h before killing. All experiments were performed concurrently. Results are expressed as means + one s.d. using 6 rats per group. Significance of differences from controls (Student's *t* test): aminophylline *v.* saline-injected controls: $^*P<0.05$; aminophylline and propranolol *v.* propranolol-injected controls: $^\dagger P<0.05$; propranolol *v.* saline-injected controls: $^\ddagger P<0.05$.

secretion (Turtle *et al.*, 1967). Figure 3 shows the concentration of total plasma tryptophan together with that of UFA and insulin. Values for brain and plasma aromatic amino acids are shown in Figure 4.

In this experiment the decrease of plasma total tryptophan was proportionately less (19%) than that found before under the same conditions (Figure 1, 28%; Table 1, 36%); as before, plasma free tryptophan concentration was essentially unaffected. The plasma concentrations of tyrosine and phenylalanine fell significantly, indicating that the fall of the plasma concentration of total tryptophan was not specific for this amino acid. These changes occurred together with a significant increase of the plasma insulin concentration. The increased ratio of brain tryptophan to plasma tryptophan was not specific for this amino acid as the ratios for the other two amino acids were also raised, significantly so in the case of tyrosine. However, this was associated with decreases in the plasma concentrations of tyrosine and phenylalanine so that their concentrations in the brain (unlike that of tryptophan) did not rise. Brain tyrosine showed a small but significant decrease.

## *Effects of aminophylline in propranolol-treated rats (Figures 3 and 4)*

The effect of $\beta$-adrenoceptor blockade on aminophylline-induced changes was investigated by giving propranolol. Propranolol alone did not alter the parameters measured except the plasma insulin concentration which fell by 50% (see Figure 3) and the ratio of brain tyrosine to plasma tyrosine which fell slightly but significantly.

Aminophylline (150 mg/kg) when given to propranolol-treated rats no longer increased plasma UFA. The plasma concentrations of total tryptophan and tyrosine fell, but that of phenylalanine was unaltered (Figure 3). The increase in the brain/plasma ratios for tryptophan and phenylalanine concentrations no longer occurred and the increase in the corresponding ratio for tyrosine was less pronounced than when aminophylline was given in the absence of propranolol. Thus propranolol largely prevented the effects of aminophylline on aromatic amino acid disposition between plasma and brain. It did not prevent the aminophylline-provoked rise in plasma insulin although the concentrations reached were somewhat lower. The insulin values were widely scattered (Figure 3). In a separate experiment propranolol decreased the raised plasma insulin concentration values 3 h after aminophylline injection from 119 ± 61 (s.d.) ($n=6$) to 53 ± 19 (s.d.) µu/ml ($n=6$). Therefore the relation between the amino acid changes and insulin remained unclear.

## *Effect of aminophylline (150 mg/kg) on aromatic amino acids in liver and muscle*

As aminophylline (150 mg/kg) altered the disposition of aromatic amino acids between plasma and brain these amino acids were estimated in other tissues 3 h

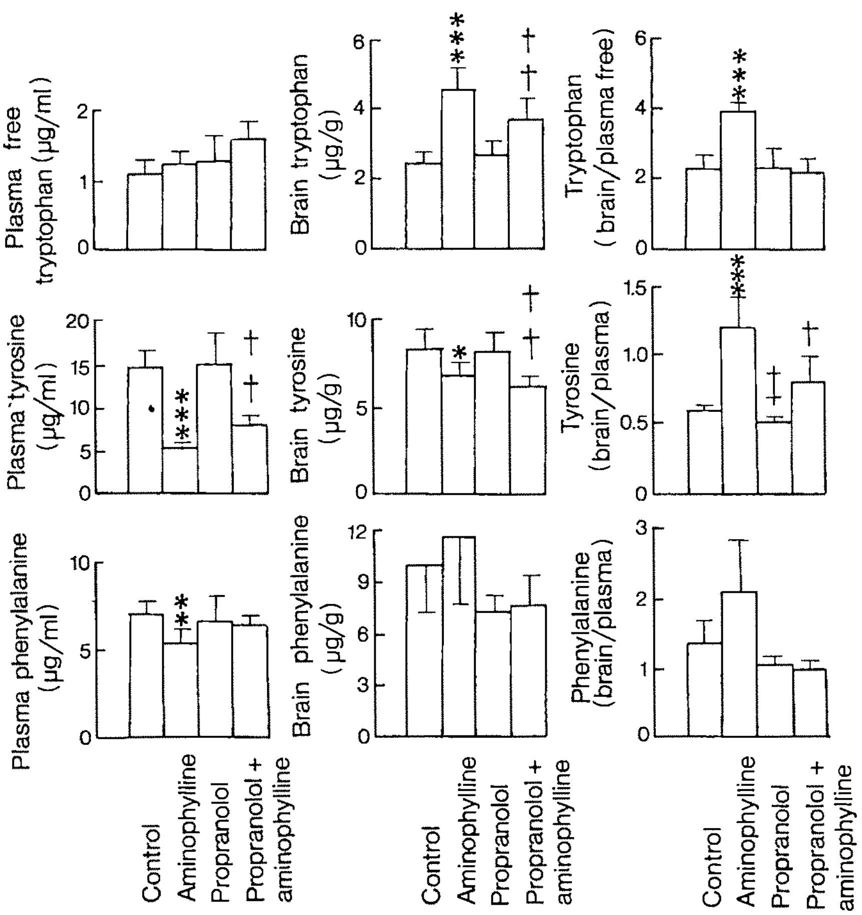


**Figure 4** Effects of aminophylline (150 mg/kg i.p.) and propranolol (2 mg/kg i.p., ×2) on plasma and brain concentrations of aromatic amino acids. Details as in Figure 3. Results are expressed as means either + or − one s.d. using 5–6 rats per group. Significance of differences from controls (Student's $t$ test): aminophylline v. saline-injected controls: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; aminophylline and propranolol v. propranolol: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$; propranolol v. saline-injected controls: $^{\ddagger}P<0.05$.

after aminophylline injection. The results in Figure 5 show that muscle tryptophan increased moderately but significantly in association with a comparable rise of plasma free tryptophan so that the muscle/plasma ratio remained unaltered. The concentrations in the liver of tryptophan, tyrosine and phenylalanine were not significantly changed. Muscle tyrosine fell significantly but as the plasma concentration fell even further the ratio, muscle tyrosine/plasma tyrosine rose significantly as did also the ratio: liver tyrosine/plasma tyrosine. These ratios were also increased for phenylalanine but not significantly.

### *Effect of aminophylline (150 mg/kg) in streptozotocin diabetic rats*

In order to clarify the role of insulin in the action of aminophylline on the disposition of aromatic amino acids the parameters shown in Figures 3 and 4 were also measured in rats made diabetic with streptozotocin (Arison, Ciaccio, Glitzer, Cassaro & Pruss, 1967) before aminophylline injection. In a preliminary experiment (Experiment 2, Table 3) streptozotocin was injected into a tail vein (65 mg/kg) 72 h before aminophylline (150 mg/kg, i.p.); 3 h later streptozotocin-treated rats had significantly lower plasma insulin values than control rats (both injected with saline). Aminophylline did not significantly increase plasma insulin in the streptozotocin-treated rats. The data obtained on the brain were not easy to interpret. Although the brain/plasma ratio for tryptophan concentrations was no longer significantly increased by aminophylline the ratios for tyrosine and phenylalanine were still significantly raised (not shown). These increases could have been due to effects of aminophylline on residual pancreatic insulin stores

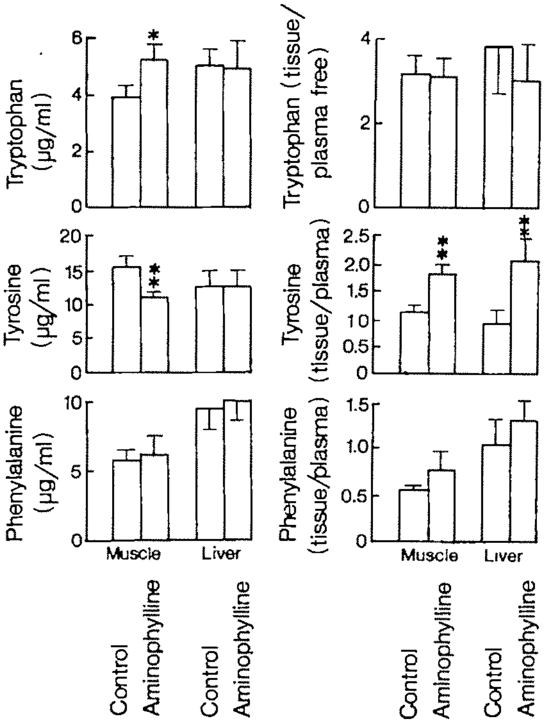


**Figure 5** Effect of aminophylline (150 mg/kg i.p.) on aromatic amino acids in muscle and liver. Aminophylline was injected 3 h before killing. Results are expressed as means either + or − one s.d. using 6 rats per group. Significance of differences from controls (Student's $t$ test): aminophylline v. saline-injected controls: $*P<0.01$, $**P<0.001$. Plasma and brain values are not shown being essentially the same as in Figures 3 and 4 except that brain phenylalanine fell by 21% ($P<0.001$) and the increase of the brain phenylalanine/plasma phenylalanine ratio was significant (+39%, $P<0.001$).

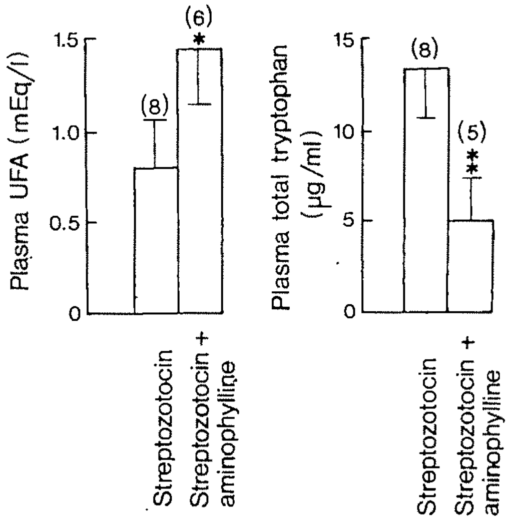


**Figure 6** Effect of aminophylline (150 mg/kg i.p.) and streptozotocin (90 mg/kg i.p.) on plasma unesterified fatty acids (UFA) and total tryptophan. Streptozotocin was injected 48 h before aminophylline and rats killed 3 h later. Results are expressed as means + one s.d. Significance of differences from streptozotocin injected controls, (Student's $t$ test): $*P<0.01$, $**P<0.001$. Insulin values are shown in Table 3, Experiment 3.

released during the first 2 h after aminophylline but no longer reflected by plasma insulin values 3 h later. This is possible because plasma insulin values of non-diabetic rats were much higher 1 h than at 3 h after aminophylline (Experiment 1, Table 3).

Therefore, in a subsequent experiment a larger dose of streptozotocin was given (90 mg/kg, i.p.). Principal

**Table 3** Effect of aminophylline on plasma insulin in normal and streptozotocin-treated rats

| | *Treatment* | *Plasma insulin (μu/ml)* | |
|---|---|---|---|
| | | *1 h*[1] | *3 h*[1] |
| Experiment 1 | Aminophylline | 380 ± 32 (6) | 119 ± 61 (6) |
| Experiment 2 | Saline | | 65 ± 11 (6) |
| | *Streptozotocin-treated rats (65 mg/kg, i.v. 72 h previously)* | | |
| | Saline | | 21 ± 11 (6)* |
| | Aminophylline | | 26 ± 9 (6) |
| Experiment 3 | *Streptozotocin-treated rats (90 mg/kg, i.p. 48–72 h previously)* | | |
| | Saline | 10 ± 3 (6) | 11 ± 6 (8) |
| | Aminophylline | 7 ± 4 (8) | 11 ± 6(5) |

None of the three experiments was performed concurrently.

Results are expressed as means ± one s.d. Nos. of determinations are shown in parentheses. [1]Rats killed at these times after intraperitoneal injection of saline or aminophylline (150 mg/kg).

* Significance of difference from rats not given streptozotocin, $P<0.001$.

results are shown in Figures 6 and 7. Plasma insulin concentrations were not altered significantly 1 and 3 h after aminophylline (Experiment 3, Table 3) and both values were lower than in the previous experiment. The plasma concentrations of UFA and free tryptophan were doubled 3 h after aminophylline. This was accompanied by a large and significant fall of the concentration of plasma total tryptophan so that the percentage of free tryptophan was increased more than five-fold. Plasma tyrosine fell significantly but much less strikingly than total tryptophan. The brain concentrations of the three amino acids were essentially unaltered (not shown) and brain/plasma ratios were no longer increased by aminophylline and in the case of tryptophan the ratio fell significantly, i.e. the rise of plasma free tryptophan was not parallelled by a rise in the brain tryptophan concentration 3 h after aminophylline injection. However, brain tryptophan may have risen at an earlier time as both 5-HT and 5-HIAA were significantly increased (5-HT, +21%, $P<0.01$; 5-HIAA, +44%, $P<0.001$). Indeed, the experiment in Table 4 indicates that 1 h after aminophylline injection brain tryptophan did rise.

In this experiment aminophylline caused a large rise in the concentration of plasma UFA (in contrast with findings 1 h after giving the same dose of the drug to non-diabetic rats, see Figure 1) and percentage free tryptophan rose proportionately although as total tryptophan fell the mean free tryptophan concentration only rose to a lesser extent and not significantly. Nevertheless brain tryptophan did increase significantly and in proportion to the increase of plasma free tryptophan.

### *Effects of other methylxanthines on brain indoles*

Table 5 shows that brain tryptophan and 5-HIAA were increased significantly by caffeine. The increase of 5-HIAA agrees with the findings of Berkowitz & Spector (1971). Theobromine at comparable doses had a similar but smaller effect and only the increased in 5-HIAA was significant.

## Discussion

The results suggest that the increases in brain 5-HT metabolism following the injection of aminophylline or other methylxanthines are the consequence of increases of brain tryptophan. Previously (Curzon &

**Table 4** Plasma unesterified fatty acid (UFA), insulin and tryptophan and brain tryptophan 1 h after injecting streptozotocin-treated rats with aminophylline

| | Plasma | | | | Brain tryptophan | Brain tryptophan / Plasma free tryptophan |
|---|---|---|---|---|---|---|
| | UFA (mEq/l) | Insulin (μu/ml) | Tryptophan (μg/ml) | | (μg/g) | |
| *Treatment* | | | Total | Free | | |
| Saline i.p. (4) | $0.44 \pm 0.23$ | $12 \pm 2$ | $17.34 \pm 2.19$ | $2.55 \pm 0.50$ | $4.13 \pm 0.63$ | $1.68 \pm 0.44$ |
| Aminophylline (150 mg/kg i.p.) (6) | $1.19 \pm 0.41^{*}$ | $8 \pm 3$ | $7.69 \pm 1.05^{***}$ | $3.63 \pm 0.91$ | $5.65 \pm 0.47^{**}$ | $1.64 \pm 0.42$ |

Streptozotocin (90 mg/kg i.p.) was given 72 h beforehand to rats which had been fasted overnight. Results are expressed as means ± one s.d. Nos. of determinations are shown in parentheses.
Significance of differences from saline-injected controls: $^{*}P<0.02$; $^{**}P<0.01$; $^{***}P<0.001$.

**Table 5** Effects of caffeine and theobromine on brain indoles

| | Brain (μg/g) | | |
|---|---|---|---|
| *Injected* | *Tryptophan* | *5-HT* | *5-HIAA* |
| Saline (i.p.) | $4.48 \pm 0.84$ | $0.54 \pm 0.12$ | $0.40 \pm 0.05$ |
| Caffeine (64 mg/kg, i.p.) | $5.80 \pm 1.09^{*}$ | $0.65 \pm 0.09$ | $0.62 \pm 0.11^{**}$ |
| Theobromine (59 mg/kg, i.p.) | $5.48 \pm 0.80$ | $0.59 \pm 0.05$ | $0.50 \pm 0.04^{**}$ |

Doses were molar equivalents of 75 mg/kg aminophylline.
Rats were killed 3 h after injection of drug. Results are expressed as means ± one s.d. for 5–6 rats/group.
Significance of difference from saline-injected controls: $^{*}P<0.05$; $^{**}P<0.01$.

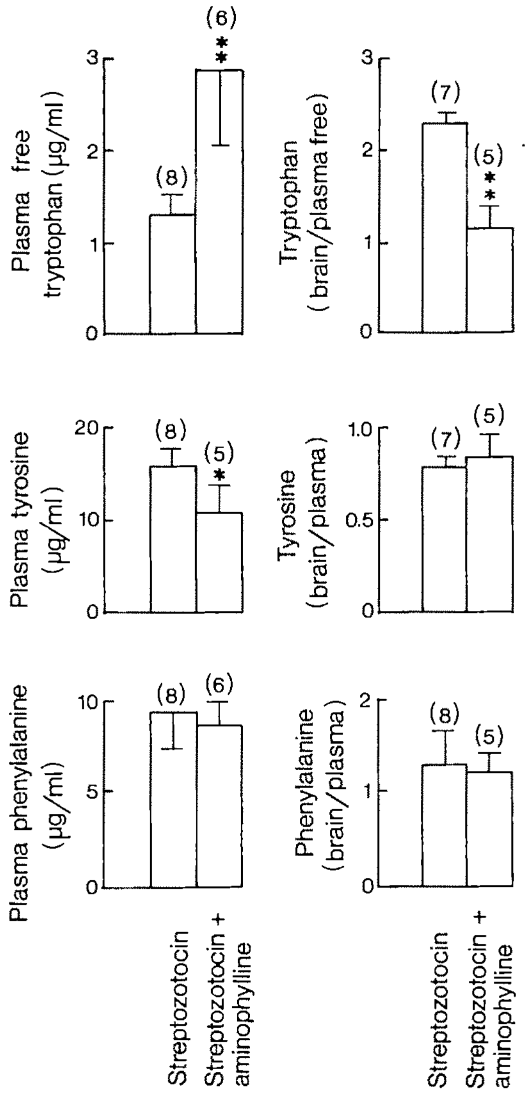


**Figure 7** Effect of aminophylline (150 mg/kg i.p.) and streptozotocin (90 mg/kp i.p.) on aromatic amino acids in plasma and brain. Details as in Figure 5. Significance of differences from streptozotocin-injected controls (Student's *t* test): $^{*}P<0.01$, $^{**}P<0.001$.

Knott, 1974) the rise in brain tryptophan caused by aminophylline has been related to the lipolytic action of the drug increasing plasma UFA so that plasma tryptophan was freed from albumin binding and became more easily available to the brain. The present more detailed study suggests that the above mechanism can explain the increase of brain tryptophan after lower doses of aminophylline ($\not>$100 mg/kg), in common with various other circumstances in which concentrations of both free plasma tryptophan and brain tryptophan alter together (Curzon *et al.*, 1973; Gessa & Tagliamonte, 1974; Knott & Curzon, 1975). However, the above mechanism does not adequately explain the increase of brain tryptophan when 150 mg/kg of aminophylline is injected.

This larger dose of aminophylline considerably increased brain tryptophan concentration whereas the plasma concentration of free tryptophan did not increase correspondingly. Thus the ratio of tryptophan concentration in brain to that in plasma was increased, suggesting increased effectiveness of tryptophan uptake by the brain. The rise of brain tryptophan concentration at the higher drug dose appeared to be specific for this amino acid as major changes in the brain concentrations of tyrosine and phenylalanine did not occur. However, the mechanism responsible for the rise of brain tryptophan is not indicated to be specific for this amino acid as brain/plasma ratios increased for all three amino acids. This agrees with the work of Petkov, Usunov & Kushev (1974) who found that theophylline injected intraperitoneally increased brain uptake of various amino acids. The fall of the plasma tyrosine concentration after aminophylline could involve increased muscle uptake of tyrosine due to the rise in insulin release (see below) as well as increased activity of tyrosine aminotransferase the synthesis of which is increased by aminophylline (Wicks, 1968) and by insulin (Gelehrter & Tomkins, 1970).

Possible mechanisms responsible for the chemical changes in the brain caused by aminophylline are suggested in Figure 8. Thus methylxanthines can increase cyclic AMP levels by releasing catecholamine (Wooten *et al.*, 1973) and thus stimulating cyclic AMP synthesis and/or by inhibiting cyclic 3′,5′-nucleotide phosphodiesterase (Butcher *et al.*, 1965) and thus preventing cyclic AMP degradation. The cyclic AMP increases in fat cells and in the pancreas result in increased lipolysis (Butcher *et al.*, 1965) and insulin secretion (Turtle *et al.*, 1967) respectively. Thus in the present study aminophylline increased both plasma UFA and insulin.

These changes can exert mutual influences. Thus insulin has antilipolytic effects (Butcher, Baird & Sutherland, 1968) while UFAs can increase insulin secretion (Malaisse & Malaisse-Lagae, 1968; Balasse & Ooms, 1973). The antilipolytic action of insulin may explain why plasma UFA did not rise until 3 h after aminophylline injection (Figure 1) and also why the increase of UFA was not directly proportional to drug dosage (Table 1). Conversely, particularly large UFA increases occurred when aminophylline was given to animals with experimental diabetes following streptozotocin treatment. That insulin secretion as well as lipolysis could be responsible for the brain tryptophan changes following aminophylline injections is indicated by previous work in which

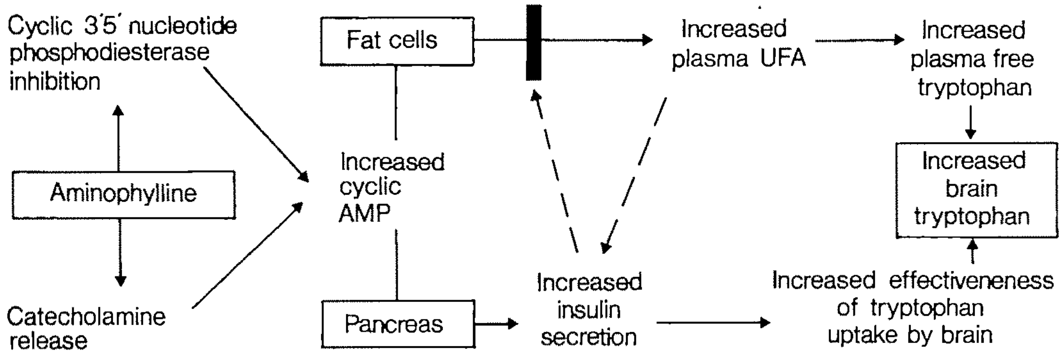

Cyclic 3'5' nucleotide phosphodiesterase inhibition
Aminophylline
Catecholamine release
Fat cells
Increased cyclic AMP
Pancreas
Increased plasma UFA
Increased plasma free tryptophan
Increased brain tryptophan
Increased insulin secretion
Increased effectiveness of tryptophan uptake by brain

**Figure 8** Relationships between aminophylline and brain tryptophan.

insulin was found to increase brain tryptophan in food-deprived rats (Fernstrom & Wurtman, 1971; Curzon & Knott, 1974) and also increased brain uptake of L-[G-$^3$H]-tryptophan (Dickerson & Pao, 1975). Furthermore, the effects of insulin and streptozotocin on the disposition of tryptophan between plasma and brain in fed rats have been explained in terms of concurrent insulin and UFA changes (Fernando *et al.*, 1976).

The increases in the plasma concentrations of insulin and UFA following aminophylline treatment are both mediated by $\beta$-adrenoceptors and such receptors seem also to be required for the effects of the drug on the disposition of tryptophan and other amino acids between plasma and brain (Figure 4) as these effects of aminophylline are prevented or decreased by propranolol. Insulin also seems to be involved in the above effect of aminophylline as it was opposed by previous streptozotocin treatment. However as aminophylline increased brain tryptophan concentration in these diabetic rats under at least some conditions (Table 4) the findings are consistent with the brain tryptophan changes not depending solely on insulin but also on plasma free tryptophan. The results in general suggest that both lipolytic and insulin secretory effects should also be considered when interpreting the effects of other drugs on brain tryptophan concentration. Whether such drugs increase lipolysis and/or insulin secretion a rise in the brain tryptophan concentration and 5-HT turnover will tend to occur. Similarly, increased lipolysis following food deprivation (Knott & Curzon, 1972) and increased insulin secretion following food intake (Fernstrom & Wurtman, 1971) can lead to increased brain tryptophan and 5-HT turnover. Thus, supplies of tryptophan to the brain for 5-HT synthesis and other purposes such as protein synthesis may be maintained in two contrasting situations.

The effectiveness of the supply of tryptophan to the brain depends on the responsiveness of $\beta$-adrenoceptors and may be opposed by stimulation of $\alpha$-adrenoceptors which decrease both insulin secretion (Turtle *et al.*, 1967) and lipolysis (Boshart, Smith, Will, Perrine & Ringler, 1961; Burns, Mohs, Langley, Yawn & Chase, 1974). Another possible limitation on the availability of tryptophan to the brain from the plasma is its uptake by other tissues. This appears to be increased by aminophylline and also by carbon tetrachloride poisoning (Knott & Curzon, 1975). Both drugs led to an increase of plasma free tryptophan and the tryptophan concentration rose not only in the brain but also in muscle (which contains much larger amino acid pools). It is worth pointing out that the aminophylline-provoked increase in insulin secretion could enhance protein synthesis in muscle (Manchester, 1970) and therefore determinations of only the non-protein bound muscle tryptophan (or other amino acids) may give a falsely low index of increased flux into muscle.

Tryptophan catabolism in the liver on the pyrrolase pathway (Curzon, 1969; Green, Woods, Knott & Curzon, 1975) may also limit its availability to the brain although in the present study aminophylline may have an opposite effect as it inhibits pyrrolase *in vivo* (Young, Oravec & Sourkes, 1974).

Although the main purpose of this work was to use aminophylline to elucidate the mechanisms by which it influences brain tryptophan and 5-HT metabolism and which may also be relevant in other circumstances, the changes found may also be involved in the behavioural effects of the drug. Thus the increased sensitivity to external stimuli following aminophylline injection appears to be mediated via catecholaminergic mechanisms (Paalzow & Paalzow, 1974) and these may well be opposed by increased 5-HT synthesis as is suggested by work on the effect of altered 5-HT synthesis on the behavioural consequences of other drugs acting on catecholamine neurones (e.g. Mabry & Campbell, 1973).

We thank the MRC for a Programme Grant. Streptozotocin was generously supplied by the Upjohn Co.

**References**

ARISON, R.N., CIACCIO, E.I., GLITZER, M.S., CASSARO, J.A. & PRUSS, M.P. (1967). Light and electron microscopy of lesions in rats rendered diabetic with streptozotocin. *Diabetes*, **16**, 51–56.

BALASSE, E.O. & OOMS, H.A. (1973). Role of plasma free fatty acids in the control of insulin secretion in man. *Diabetologia*, **9**, 145–151.

BERKOWITZ, B.A. & SPECTOR, S. (1971). The effect of caffeine and theophylline on the disposition of brain serotonin in the rat. *Eur. J. Pharmac.*, **16**, 322–325.

BOSHART, C.R., SMITH, T.C., WILL, L., PERRINE, J. & RINGLER, I. (1961). Effect of phentolamine on body fat. *Nature, Lond.*, **189** 405–406.

BURNS, T.W., MOHS, J.M., LANGLEY, P.E., YAWN, R. & CHASE, G.R. (1974). Regulation of human lipolysis: *In vivo* observations on the role of adrenergic receptors. *J. clin. Invest.*, **53**, 338–341.

BUTCHER, R.W., BAIRD, C.E. & SUTHERLAND, E.W. (1968). Effects of lipolytic and antilipolytic substances on adenosine 3′,5′-monophosphate levels in isolated fat cells. *J. biol. Chem.*, **243**, 1705–1712.

BUTCHER, R.W., HO, R.J., MENG, H.C. & SUTHERLAND, E.W. (1965). Adenosine 3′,5′-monophosphate in biological materials. II. The measurement of adenosine 3′,5′-monophosphate in tissues and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine. *J. biol. Chem.*, **240**, 4515–4523.

CURZON, G. (1969). Tryptophan pyrrolase — a biochemical factor in depressive illness? *Br. J. Psychiat.*, **115**, 1367–1374.

CURZON, G., FRIEDEL, J. & KNOTT, P.J. (1973). The effects of fatty acids on the binding of tryptophan to plasma protein. *Nature*, **242**, 198–200.

CURZON, G., JOSEPH, M.H. & KNOTT, P.J. (1972). Effects of immobilization and food deprivation on rat brain tryptophan metabolism. *J. Neurochem.*, **19**, 1967–1974.

CURZON, G., KANTAMANENI, B.D., WINCH, J., ROJAS-BUENO, A., MURRAY-LYON, I.M. & WILLIAMS, R. (1973). Plasma and brain tryptophan changes in experimental acute hepatic failure. *J. Neurochem.*, **21**, 137–146.

CURZON, G. & KNOTT., P.J. (1974). Effects on plasma and brain tryptophan in the rat of drugs and hormones that influence the concentration of unesterified fatty acid in the plasma. *Br. J. Pharmac.*, **50**, 197–204.

CURZON, G., KNOTT, P.J., MURRAY-LYON, I.M., RECORD, C.O. & WILLIAMS, R. (1975). Disturbed brain tryptophan metabolism in hepatic coma. *Lancet*, **i**, 1092–1093.

DALTON, C., VAN TRABERT, T.C. & DWYER, J.X. (1970). Hypolipidemic effects of nicotinamide in the rat arising from its transformation to nicotinic acid. In *Metabolic Effects of Nicotinic Acid and its Derivatives*, ed., Gey, K.F. & Carlson, L.A. pp. 65–75. Berne: Huber.

DICKERSON, J.W.T. & PAO, S.K. (1975). The effect of a low protein diet and exogenous insulin on brain tryptophan and its metabolites in the weanling rat. *J. Neurochem.*, **25**, 559–564.

ECCLESTON, D., ASHCROFT, G.W. & CRAWFORD, T.B.B. (1965). 5-Hydroxyindole metabolism in rat. A study of intermediate metabolism using the technique of tryptophan loading. *J. Neurochem.*, **12**, 493–503.

FERNANDO, J.C.R., KNOTT, P.J. & CURZON, G. (1976). The relevance of both plasma free tryptophan and insulin to rat brain tryptophan concentration. *J. Neurochem.* (in press).

FERNSTROM, J.D. & WURTMAN, R.J. (1971). Brain serotonin content: increase following ingestion of carbohydrate diet. *Science*, **174**, 1023–1025.

FRIEDMAN, P.A., KAPPELMAN, A.G. & KAUFMAN, S. (1972). Partial purification and characterisation of tryptophan hydroxylase from rabbit hindbrain. *J. biol. Chem.*, **247**, 4165–4173.

GEREHRTER, T.D. & TOMKINS, G.M. (1970). Postranscriptional control of tyrosine aminotransferase synthesis by insulin. *Proc. Nat. Acad. Sci.*, **66**, 390–397.

GESSA, G.L. & TAGLIAMONTE, A. (1974). Serum free tryptophan: control of brain concentrations of tryptophan and of synthesis of 5-hydroxytryptamine. In *Aromatic Amino Acids in the Brain. Ciba Foundation Symposium*, **22**, pp. 207–216. Amsterdam: Elsevier.

GREEN, A.R., WOODS, F., KNOTT, P.J. & CURZON, G. (1975). Factors influencing effect of hydrocortisone on rat brain tryptophan metabolism. *Nature, Lond.*, **255**, 170.

HERY, R., ROUER, E., KAN, J.P. & GLOWINSKI, J. (1974). The major role of the tryptophan active transport system in the diurnal variations of 5-hydroxytryptamine synthesis in the rat brain. *Adv. biochem. Psychopharmac.*, **11**, 163–167.

KIELY, M. & SOURKES, T.L. (1972). Transport of L-tryptophan into slices of rat cerebral cortex. *J. Neurochem.*, **19**, 2863–2872.

KNOTT, P.J. & CURZON, G. (1972). Free tryptophan in plasma and brain tryptophan metabolism. *Nature*, **239**, 452–453.

KNOTT, P.J. & CURZON, G. (1975). Tryptophan and tyrosine disposition and brain tryptophan metabolism in acute carbon tetrachloride poisoning. *Biochem. Pharmac.*, **24**, 963–966.

MABRY, P.D. & CAMPBELL, B.A. (1973). Serotonergic inhibition of catecholamine induced behavioural arousal. *Brain Res.*, **49**, 381–391.

MADRAS, B.K., COHEN, E.L., MESSING, R., MUNRO, H.N. & WURTMAN, R.J. (1974). Relevance of free tryptophan in serum to tissue tryptophan concentrations. *Metabolism*, **23**, 1107–1116.

MALAISSE, W.F. & MALAISSE-LAGAE. (1968). Stimulation of insulin secretion by non-carbohydrate metabolites. *J. lab. clin. Med.*, **72**, 438–448.

MANCHESTER, K.L. (1970). The control by insulin of amino acid accumulation in muscle. *Biochem. J.*, **117**, 457–465.

McCAMAN, M.W. & ROBINS, E. (1962). Fluorimetric method for the determination of phenylalanine in serum. *J. lab. clin. Med.*, **59**, 885–890.

MUNRO, H.N., FERNSTROM, J.D. & WURTMAN, R.J. (1975). Insulin, plasma amino acid imbalance, and hepatic coma. *Lancet*, **i**, 722–724.

OLDENDORF, W.H. (1971). Brain uptake of radiolabelled amino acids, amines and hexoses after arterial injection. *Amer. J. Physiol.*, **221**, 1629–1639.

PAALZOW, G. & PAALZOW, L. (1974). Theophylline increased sensitivity to nociceptive stimulation and regional turnover of rat brain 5-HT, noradrenaline and dopamine. *Acta Pharmac. tox.*, **34**, 157–173.

PETKOV, V., USUNOV, P. & KUSHEV, V. (1974). The role of 3′,5′-AMP system in amino acid transport across the blood-brain barrier. *Acta biol. med. germ.*, **33**, 371–383.

TAGLIAMONTE, A., BIGGIO, G., VARGIU, L. & GESSA, G.L. (1973). Free tryptophan in serum controls brain tryptophan level and serotonin synthesis. *Life Sciences*, **12**, 277–287.

TAGLIAMONTE, A., TAGLIAMONTE, P., FORN, J., PEREZ-CRUET, J., KRISHNA, G. & GESSA, G.L. (1971). Stimulation of brain serotonin synthesis by dibutyryl-cyclic AMP in rats. *J. Neurochem.*, **18**, 1191–1196.

TURTLE, J.R., LITTLETON, G.K. & KIPNIS, D.M. (1967). Stimulation of insulin secretion by theophylline. *Nature, Lond.*, **213**, 727–728.

WICKS, W.D. (1968). Tyrosine-α-ketoglutarate transaminase: induction by epinephrine and adenosine-3′,5′-cyclic phosphate. *Science*, **160**, 997–998.

WOOTEN, G.F., THOA, N.B., KOPIN, I.J. & AXELROD, J. (1973). Enhanced release of dopamine- beta hydroxylase and norepinephrine from sympathetic nerves by dibutyryl cyclic adenosine 3′,5′-monophosphate and theophylline. *Molec. Pharmac.*, **9**, 178–183.

YOUNG, S.N., ORAVEC, M. & SOURKES, T.L. (1974). The effect of theophylline on tryptophan pyrrolase in the hypophysectomized rat and some observations on the validity of tryptophan pyrrolase assays. *J. biol. Chem.*, **249**, 3932–3936.

*(Received March 18, 1976.*
*Revised May 19, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 547–551

# THE EFFECT OF A NEW ANTI-INFLAMMATORY DRUG, FLURBIPROFEN, ON THE RESPIRATORY, HAEMODYNAMIC AND METABOLIC RESPONSES TO *E. coli* ENDOTOXIN SHOCK IN THE CAT

J.R. PARRATT & R.M. STURGESS
The Department of Physiology and Pharmacology, University of
Strathclyde, Royal College, 204 George Street, Glasgow, G1 1XW

1 The intravenous administration of *E. coli* endotoxin (2.0 mg/kg) in cats anaesthetized with sodium pentobarbitone resulted in immediate pulmonary hypertension and reductions in lung compliance and systemic arterial $P_{O_2}$. These effects were abolished, or greatly reduced, by the prior intravenous administration of flurbiprofen in doses (100 and 250 μg/kg and 1.0 mg/kg) which were devoid of cardiovascular or metabolic effects. Flurbiprofen is thus the most active antipyretic-analgesic drug so far examined in this experimental model.

2 Production of lactate, characteristic of the severe, secondary endotoxin shock phase, was delayed only by the highest dose of flurbiprofen; hypotension, hypoglycaemia and the reduction in cardiac output which occurs during this phase, were unaffected.

3 These findings are discussed with reference to the treatment of the 'shocked lung' syndrome of human septicaemia.

## Introduction

Marked pulmonary changes occur in patients with septic shock; there is pulmonary vascular obstruction (perhaps due, in part, to emboli, intravascular coagulation and platelet aggregation), interstitial pulmonary oedema, a reduction in lung compliance and disturbances in the ventilation-perfusion relationship (Pontoppidan, Geffin & Laver, 1971). These changes give rise to the so-called 'shock lung' syndrome. Similar changes have been described in rabbits following the administration of *Salmonella typhi* endotoxin (Gemer, Hayes, Ishikawa, Cuevas & Hirsch, 1973).

In a previous study (Parratt & Sturgess, 1974) we showed that indomethacin, in a single intravenous dose of 10 mg/kg, prevented the pulmonary hypertension and oedema that follows the administration of *E. coli* endotoxin in the cat. It also delayed the onset of the secondary shock phase, which is characterized in this species by systemic hypotension, a reduction in cardiac output and an increasingly severe metabolic acidosis (Parratt, 1973). However, one problem with using indomethacin in this experimental model is that it can cause substantial myocardial depression (Parratt & Sturgess, 1974). Although it is not known whether the pulmonary changes that occur in septic shock are modified by drugs like indomethacin it seemed worthwhile exploring the effect of other antipyretic-analgesic drugs on the pulmonary effects of endotoxin in the cat; such drugs might be more effective than indomethacin and cause less myocardial depression, an important consideration since myocardial function is often depressed in septic shock (Siegel & Fabian, 1967). This paper describes the effects, in experimental endotoxin shock, of flurbiprofen (2-(2-fluoro-4-biphenylyl)-propionic acid) a potent, orally active non-steroidal anti-inflammatory drug (Glenn, Rohloff, Bowman & Lyster, 1973; Adams, McCullough & Nicholson, 1975) which recently has been shown to be extremely active in inhibiting human platelet aggregation (Nishizawa, Wynalda, Suydam & Molony, 1973; Davies, Lederer, Spencer & McNicol, 1974; Sim, McCraw & Sim, 1975).

## Methods

Cats of either sex were anaesthetized with sodium pentobarbitone (36 mg/kg body weight, intraperitoneally), with additional 6 mg doses intravenously given as required throughout the course of the experiment to maintain anaesthesia. A femoral vein was cannulated for the administration of endotoxin and drugs, and 300 units/kg of heparin was given intravenously before proceeding with further surgery. Systemic arterial pressure was recorded with a capacitance transducer (Elema-Schönander EMT 35) from a catheter inserted, by way of the right carotid artery, such that the tip lay in the aortic arch. Mean pressure was obtained by electronic integration and aortic d*P*/d*t* was continuously determined with an Elema-Schönander differentiator. Pulmonary artery ('downstream') pressure was recorded, following a left thoracotomy, via a needle-tipped catheter inserted directly through the wall of the artery, and connected to a Statham P23Db transducer.

In all cats the trachea was cannulated with a glass T-piece cannula and the animals respired with room air using a Palmer positive pressure ventilation pump at a rate of 20 per minute. The stroke volume was adjusted to approximately 20 ml/kg body weight, in order to give an arterial $Po_2$ of over 80 mmHg, and was usually within the range 40–60 ml. In order to measure the intratracheal pressure, a polythene catheter was sealed into the side arm of the cannula, and connected to a Statham P23Db transducer. At various times during these experiments, in order to assess changes in airway dynamic compliance, the respiratory stroke volume was readjusted to different levels, and the corresponding change in intratracheal pressure measured; at least 20–30 min were allowed to elapse after this procedure before any attempt was made to record cardiac output or to sample blood.

Cardiac output was measured by thermodilution as previously described (Parratt, 1973).

Systemic arterial pressure, pulmonary artery pressure, aortic d*P*/d*t*, and the electrocardiogram (standard lead II) were recorded simultaneously on a display oscilloscope (Racal Instruments) and on an ink-jet writing recorder (Elema-Schönander mingograph 81).

Blood samples (1.0–2.0 ml) were withdrawn from the arterial catheter to be analysed for $O_2$ and $CO_2$ tensions, and pH, using appropriately calibrated systems (Radiometer, Copenhagen). The pH electrode was calibrated by means of standard buffers, and the $O_2$ and $CO_2$ electrodes with gas mixtures, the concentrations of which had been measured with a modified Lloyd-Haldane apparatus. $O_2$ and $CO_2$ tensions, and pH, were corrected for any temperature difference between the animals' mid-oesphageal temperature and the electrode temperature (usually 37.2°C), by means of the blood gas calculator described by Severinghaus (1966). Arterial lactate and glucose were measured as described previously (Parratt & Sturgess, 1974).

After a period of stabilization, the cats were given *E. coli* endotoxin (Difco Laboratories 055:B5) suspended in 0.9% w/v sodium chloride solution (saline), and administered slowly by intravenous injection in a dose of 2 mg/kg. This dose kills 92% of the animals within 6 h (Parratt & Sturgess, unpublished results). Three groups, each of five cats, were administered sodium flurbiprofen, in doses of 100 µg/kg, 250 µg/kg, and 1 mg/kg respectively, 30 min before the endotoxin; the responses were compared with those of a group of eight cats given endotoxin alone. Measurements were made of the haemodynamic and metabolic parameters until either the animals died, or the experiments were terminated 6 h post-endotoxin.

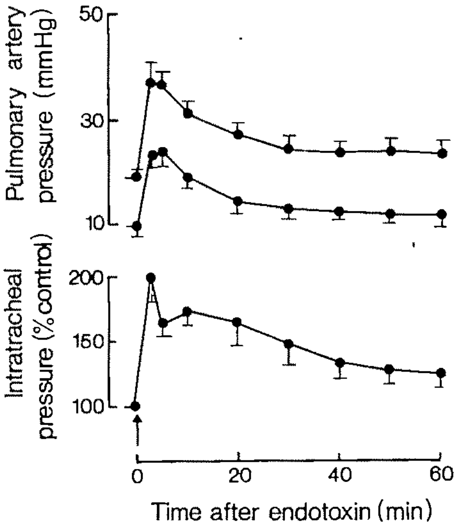


**Figure 1** The effects of *E. coli* endotoxin (2 mg/kg) on pulmonary arterial and intratracheal pressures in the cat. The initial response to endotoxin consisted of marked pulmonary hypertension, and an increase in intratracheal pressure at a constant volume lung inflation (i.e. a decreased compliance). Values are means, vertical lines show s.e. means.

## Results

### *Responses to* E. coli *endotoxin*

Within 30 s of the completion of the injection, and often whilst it was still in progress, pulmonary artery pressure rose, attaining a maximum after 3–5 min (Figure 1). Thereafter, pulmonary artery pressure tended to return slowly towards normal, although it remained slightly, but significantly, elevated up to 2 h after endotoxin administration. The rise in pulmonary pressure preceded by a few seconds a parallel increase in intratracheal pressure (Figure 1). The maximum, however, occurred somewhat earlier than that of the pulmonary pressure; expressing intratracheal pressure as a percentage of the pre-endotoxin value, it was 199 ± 23% 2 min after endotoxin, and 172 ± 21% 4 min afterwards. Endotoxin greatly reduced pulmonary dynamic compliance (as assessed 30 and 60 min after the injection from curves relating intratracheal pressure to respiratory stroke volume) and this is illustrated in Figure 2. These pulmonary

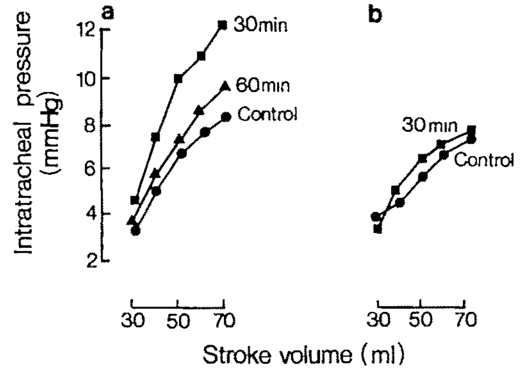


**Figure 2** (a) Pulmonary compliance curves before (0), and 30 and 60 min after, the administration of endotoxin. Each curve is the mean of from 3 to 5 experiments in different animals. An increase in intratracheal pressure at given fixed respiratory stroke volumes indicates a reduction in pulmonary compliance. (b) The effect of endotoxin in 5 cats pretreated with sodium flurbiprofen (100 µg/kg); the endotoxin-induced reduction in compliance is almost completely prevented.

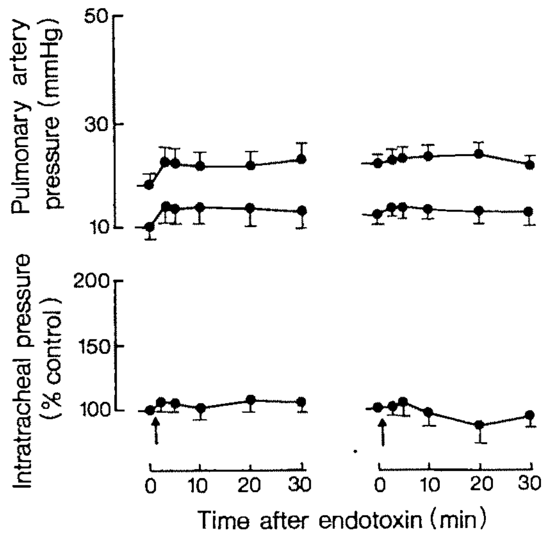


**Figure 3** Modification by pretreatment with sodium flurbiprofen (100 µg/kg on the left and 250 µg/kg on the right) of the responses to endotoxin (at the arrow). There is an almost complete abolition of the pulmonary effects (compare endotoxin-alone responses in Figure 1).

changes also affected gas exchange; during the initial hour following endotoxin administration the systemic arterial $P\text{O}_2$ was reduced by 22 ± 5.7 mmHg.

Beginning a few seconds after the start of the rise in pulmonary artery pressure, and usually after that of the intratracheal pressure, carotid artery pressure fell rapidly, often to levels below 50 mmHg. This was only a transient effect, much shorter in duration than that on the pulmonary circulation. Carotid artery d$P$/d$t$ *max* showed a similar transient decrease. Heart rate decreased with the fall in systemic arterial pressure, and ventricular extrasystoles were observed in a number of experiments. These parameters returned to control values within 3–4 min, but there was a decline in cardiac output and in systemic pressure over the next 3–5 h, results similar to those described in detail in a previous publication (Parratt & Sturgess, 1974).

Endotoxin administration resulted in a substantial metabolic acidosis, the arterial pH falling from a pre-endotoxin value of 7.532 ± 0.035 to 7.246 ± 0.034 at 1 h ($P < 0.001$), to 7.135 ± 0.056 at 2 h ($P < 0.001$) and 7.140 ± 0.082 at 3 h ($P < 0.001$). This was associated with a two- to eight-fold increase in arterial blood lactate concentrations; e.g. 5.4 ± 0.5 mg/100 ml pre-endotoxin, to 39.7 ± 4.6 mg/100 ml at 2 hours. Blood glucose progressively declined after endotoxin administration (115 ± 11 mg/100 ml control; 97 ± 20 mg/100 ml at 2 h (NS) and 59 ± 21 mg/100 ml ($P < 0.005$) at 3 hours).

### *Direct effects of sodium flurbiprofen*

Sodium flurbiprofen (0.1, 0.25, and 1.0 mg/kg) had no significant effect on systemic arterial pressure and d$P$/d$t$ *max*, pulmonary artery pressure, heart rate, cardiac output, intratracheal pressure or lung compliance. Flurbiprofen was also without a significant effect on arterial lactate, glucose, pH, or $P\text{O}_2$. These results are in contrast to those previously obtained with indomethacin (Parratt & Sturgess, 1974), which caused substantial reductions in systemic and pulmonary artery blood pressures, and in cardiac output over a 30 min period.

### *The effects of sodium flurbiprofen on the responses to endotoxin*

The acute response to endotoxin was markedly reduced by all three doses of sodium flurbiprofen. There were only transient decreases of carotid artery pressure and d$P$/d$t$, which were much less than in those animals given endotoxin alone. The pulmonary responses to endotoxin were almost completely abolished, even with a dose of sodium flurbiprofen as low as 100 µg/kg (Figure 3). For example, in the endotoxin control animals, increases in pulmonary artery pressure of 20–30 mmHg were observed, whilst in those given flurbiprofen before the endotoxin, the changes were usually less than 5 mmHg. The reduction in pulmonary dynamic compliance, which is a characteristic of the feline response to endotoxin, was abolished by pretreatment with sodium flurbiprofen (Figure 2) and there was no reduction in arterial $P\text{O}_2$ (Table 1).

Although flurbiprofen greatly reduced the initial response of cats to endotoxin, it had little significant effect on the secondary, delayed, shock phase (characterized in this species by systemic hypotension, decreased cardiac output and a pronounced metabolic acidosis; Parratt, 1973; Parratt & Sturgess, 1974). In the control group the carotid blood pressure had fallen from 118 ± 8 mmHg (systolic) and 89 ± 9 mmHg (diastolic) to 89 ± 2 mmHg and 49 ± 10 mmHg 3 h after endotoxin ($P < 0.01$) and the heart rate had increased from 201 ± 16 beats/min to 233 ± 4 beats/minute. The corresponding values 3 h after endotoxin in the flurbiprofen group (1.0 mg/kg) were 85 ± 15 mmHg, 57 ± 15 mmHg and 199 ± 22 beats/minute. However, there was some evidence that the metabolic deterioration induced by endotoxin was slightly delayed in the cats given this dose of flurbiprofen (Table 1). Thus at 1 h the pH in the endotoxin-alone group was 7.246 ± 0.034 (lactate level 24.2 ± 2.2 mg/100 ml) compared with 7.397 ± 0.066 (lactate level 13.1 ± 3.3 mg/100 ml) in the flurbiprofen group ($P < 0.01$). At 3 h there was no significant difference between the two groups and flurbiprofen did not improve survival rate assessed at 4 h or 6 hours. Endotoxin-induced hypoglycaemia which has recently been correlated with lethality in endotoxin shock (Hinshaw, Benjamin, Coalson, Elkins, Taylor, Price, Smith & Greenfield, 1975) was unaffected by pretreatment with flurbiprofen (Table 1).

## Discussion

In the cat the acute administration of *E. coli* endotoxin results in marked pulmonary hypertension (Parratt, 1973 and Figure 1 of the present paper) and this has now been shown to be accompanied by a considerable increase in intratracheal pressure at a fixed respiratory volume (Figure 1). A study of the relationship between intratracheal pressure and respiratory volume 30 min after endotoxin administration (when the end-inspiratory intratracheal pressure was still nearly 150% of control) clearly showed reductions in lung dynamic compliance (Figure 2). There was obvious respiratory distress and pulmonary oedema. This resulted in a decrease in systemic arterial $Po_2$ even at 1 h after endotoxin when pulmonary arterial and intratracheal pressures were only slightly, though significantly, elevated. There is some evidence that these pulmonary changes are mediated partly through the release of a prostaglandin (Parratt & Sturgess, 1975a). Flurbiprofen reduced these endotoxin-induced pulmonary changes in doses as low as 100 μg/kg (Figure 3). This makes the drug several hundred times more active than indomethacin (Parratt & Sturgess, 1974) and about ten times more active than sodium meclofenamate (Parratt & Sturgess, 1975b) in this experimental model. This is in line with the results of

**Table 1** Effect of sodium flurbiprofen (1 mg/kg) on the metabolic response to *E. coli* endotoxin

| *Time after endotoxin* | *0 h* | *1 h* | *2 h* | *3 h* | *4 h* |
|---|---|---|---|---|---|
| Arterial blood: | | | | | |
| pH (units) | 7.538 ± 0.027 | 7.397 ± 0.066† | 7.307 ± 0.094*† | 7.215 ± 0.173* | 7.075 ± 0.151* |
| $Po_2$ (mmHg) | 91.4 ± 4.0 | 95.4 ± 6.8** | 99.0 ± 8.1 | 96.7 ± 13.8 | 103.4 ± 16.7 |
| Lactate (mg/100 ml) | 10.30 ± 4.04 | 13.06 ± 3.29† | 29.41 ± 7.84* | 31.53 ± 10.24 | 43.97 ± 9.20* |
| Glucose (mg/100 ml) | 92 ± 17 | 93 ± 30 | 72 ± 21 | 57 ± 11* | 33 ± 15* |

** Rise in $Po_2$ during first hour = 3.1 ± 3.5 mmHg ($P < 0.01$ *vs.* endotoxin alone where there was a reduction of 22.6 ± 5.7 mmHg).
* $P < 0.05$ difference from pre-endotoxin (0 h) value; † $P < 0.05$ difference between treated and non-treated animals.

other tests (anti-inflammatory, analgesic and antipyretic; Adams *et al.*, 1975). Furthermore, the protection afforded against the endotoxin-induced pulmonary changes was obtained with doses that had no depressant effects on the cardiovascular system. There appear to be two, possibly related, explanations for this protective effect; inhibition of prostaglandin synthetase (and hence prevention of the synthesis and release of a pulmonary constrictor prostaglandin) and prevention of endotoxin-induced platelet aggregation. It is of interest in this connection that the activity of flurbiprofen in inhibiting collagen-induced platelet aggregation (Nishizawa *et al.*, 1973) approaches that of prostaglandin $E_1$, the most potent known inhibitor of platelet aggregation.

Despite the protection afforded by flurbiprofen in the initial (pulmonary) phase of endotoxin shock, there appeared to be little modification of the secondary shock phase, apart from a delay in the appearance of the metabolic consequences of endotoxaemia (Table 1). Flurbiprofen did not prevent the onset of the metabolic acidosis nor the pronounced hypoglycaemia, which has been shown by Hinshaw's group to be a relatively early event in the shock process and which is correlated with the progressive development of the irreversible state (Hinshaw *et al.*, 1975). When administered before endotoxin, flurbiprofen clearly had no effect on the severity or development of the delayed shock process. A point of difference with results obtained with indomethacin (Parratt & Sturgess, 1974) and with sodium meclofenamate (Parratt & Sturgess, 1975c) was the absence of any sustained rise in blood pressure following the administration of endotoxin, an effect ascribed to inhibition of prostaglandin synthetase.

Although pulmonary changes are often marked in patients with septic shock (reduction in lung compliance and increase in pulmonary resistance) we know of no pathological studies of the lesions that result from endotoxin administration in the cat. Such a comparative study of the pulmonary lesions in experimental endotoxin shock and in septic shock patients has recently been started (Clements, Parratt & Rodger, unpublished). Clearly, if the lesions are similar and have a common pathogenesis (active airway constriction by a prostaglandin $F_{2\alpha}$-like mediator or mechanical obstruction with aggregating platelets) there is a case for an investigation of flurbiprofen as an adjunct to the therapy of septic shock.

This work was supported by the Medical Research Council, R.M.S. was a Strathclyde University open scholar. We are grateful to Dr Stewart Adams (Boots Company Ltd.) for a generous supply of flurbiprofen.

## References

ADAMS, S.S., McCULLOUGH, K.F. & NICHOLSON, J.S. (1975). Some biological properties of flurbiprofen, an anti-inflammatory, analgesic and antipyretic agent. *Arzneim.-Forsch.*, **25**, 1786–1791.

DAVIES, T., LEDERER, D.A., SPENCER, A.A. & McNICOL, G.P. (1974). The effect of flurbiprofen (2-(2-fluoro-4-biphenylyl) propionic acid) on platelet function and blood coagulation. *Thrombosis Res.*, , 667–683.

GEMER, M., HAYES, J.A., ISHIKAWA, S., CUEVAS, P. & HIRSCH, E.F. (1973). Functional and structural changes in the lungs of rabbits with endotoxemia. *Surgery, Gynec., Obstet.*, **137**, 975–979.

GLENN, E.M., ROHLOFF, N., BOWMAN, B.J. & LYSTER, S.C. (1973). The pharmacology of 2-(2-fluoro-4-biphenylyl) propionic acid (flurbiprofen), a potent non-steroidal anti-inflammatory drug. *Agents & Actions*, **3/4**, 577–588.

HINSHAW, L.B., BENJAMIN, B., COALSON, J.J., ELKINS, R.C., TAYLOR, F.B., PRICE, J.T., SMITH, C.W. & GREENFIELD, L.J. (1975). Hypoglycaemia in lethal septic shock in subhuman primates. *Circulat. Shock*, **2**, 197–208.

NISHIZAWA, E.E., WYNALDA, D.J., SUYDAM, D.E. & MOLONY, B.A. (1973). Flurbiprofen, a new potent inhibitor of platelet aggregation. *Thrombosis Res.*, **3**, 577–588.

PARRATT, J.R. (1973). Myocardial and circulatory effects of *E. coli* endotoxin; modification of responses to catecholamines. *Br. J. Pharmac.*, **47**, 12–25.

PARRATT, J.R. & STURGESS, R.M. (1974). The effect of indomethacin on the cardiovascular and metabolic responses to *E. coli* endotoxin in the cat. *Br. J. Pharmac.*, **50**, 177–183.

PARRATT, J.R. & STURGESS, R.M. (1975a). Evidence that prostaglandin release mediates pulmonary vasoconstriction induced by *E. coli* endotoxin. *J. Physiol., Lond.*, **246**, 79–80P.

PARRATT, J.R. & STURGESS, R.M. (1975b). The protective effect of sodium meclofenamate in experimental endotoxin shock. *Br. J. Pharmac.*, **53**, 466P.

PARRATT, J.R. & STURGESS, R.M. (1975c). The effects of the repeated administration of sodium meclofenamate, an inhibitor of prostaglandin synthetase, in feline endotoxin shock. *Circulat. Shock*, **2**, 301–310.

PONTOPPIDAN, H., GEFFIN, B. & LAVER, M.B. (1971). Respiratory failure in septic shock. In *Septic Shock in Man*, ed. Hershey, S.G., Del Guercio, L.R.M. & McConn, R. pp. 37–45. Boston: Little, Brown.

SEVERINGHAUS, J.W. (1966). Blood gas calculator. *J. appl. Physiol.*, **21**, 1108–1116.

SIEGEL, J.H. & FABIAN, M. (1967). Therapeutic advantages of an inotropic vasodilator in endotoxin shock. *J. Am. med. Ass.*, **200**, 120–128.

SIM, A.K., McCRAW, A.P. & SIM, M.F. (1975). An evaluation of the effects of flurbiprofen (2-(2-fluoro-4-biphenylyl) propionic acid) on platelet behaviour. *Thrombosis Res.*, **7**, 655–668.

*(Received March 22, 1976.*
*Revised May 31, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 553–559

# MYOCARDIAL UPTAKE OF DIGOXIN IN CHRONICALLY DIGITALIZED DOGS

EVA STEINESS & N. VALENTIN
Medical Department B, Surgical Department R, Division of Cardiac Surgery, Rigshospitalet and Department of Pharmacology, University of Copenhagen, Denmark

1 The time course of myocardial uptake of digoxin, increase in contractility and changes in myocardial potassium concentration was studied for 90 min following an intravenous digoxin dose to long-term digitalized dogs.

2 Nineteen dogs were investigated by the use of a biopsy technique which allowed sampling before and after administration of digoxin.

3 Ten minutes after administration of digoxin the myocardial concentration increased from 60 to 306 nmol/kg tissue; the myocardial concentration of digoxin was significantly lower (250 nmol/kg tissue) after 30 min and then increased again.

4 The transmural myocardial distribution of digoxin was uniform before and 90 min after administration of digoxin in long-term digitalized dogs but at 10 min after administration, both the subepicardial and the subendocardial concentration of digoxin were significantly lower than that of the mesocardial layer.

5 During the first 10 min the $dp/dt_{max}$ increased to 135% of the control level. The increase remained unchanged during the rest of the study.

6 Myocardial potassium decreased throughout the study.

7 The M-configuration of the myocardial uptake curve and the non-uniformity of myocardial distribution of digoxin observed at 10 min after administrating digoxin to long-term digitalized dogs indicate that the distribution of myocardial blood flow may be changed during chronic digitalization.

## Introduction

Studies of the myocardial uptake of digoxin in dogs with intact circulation have demonstrated a very fast myocardial uptake of digoxin and an initial correspondence between the myocardial digoxin concentration and the positive inotropic effect (Doherty & Perkins, 1966; Goldman, Deutscher, Schweizer & Harrison, 1972; Deutscher, Harrison & Goldman, 1972; Steiness & Valentin, 1976). In previous work it was indicated that the positive inotropic effect is secondary to changes in the intracellular concentrations of sodium and potassium induced by digitalis (Langer, 1972).

On the other hand, both *in vitro* and *in vivo* studies have demonstrated digitalis-induced positive inotropic effect without simultaneous inhibition of the Na-K membrane ATPase (Klaus, Kuschinsky & Lüllmann, 1961; Grupp & Charles, 1964; Lüllmann & Ravens, 1973; Okita, Richardson & Roth-Schechter, 1973; Steiness & Valentin, 1976). These observations, therefore, suggested the existence of at least two different myocardial receptor sites for digitalis. One of these, localized on the cell membrane, is responsible for the inhibition of the Na-K membrane ATPase (Schwartz, Matsui & Laughter, 1968). The localization of the other receptor is unknown, but could be correlated to the liberation and the rebinding of ionized calcium during the contractile cycle and thus be responsible for the positive inotropic effect.

These problems have been investigated in animals without previous long-term digitalization. The purpose of the present study was to see whether long-term treatment with digoxin influences the rate of the myocardial digoxin uptake, the digoxin-induced positive inotropic effect and the digoxin-induced myocardial loss of potassium produced by intravenous digoxin.

## Methods

Nineteen dogs, ranging in weight from 17.5 to 26 kg (median 21 kg), were studied. Eleven of the dogs received 0.25 mg digoxin twice daily during the last 8 days before the study. The last dose was given not later than 12 h before the study to ensure an equilibrium between the plasma concentration of digoxin and the myocardium (steady state). These dogs were divided into two groups. Group 1: In eight dogs series of myocardial biopsies were taken before and after an intravenous dose of digoxin (0.05 mg/kg body weight). Group 2: In three dogs myocardial biopsies were only taken before and 90 min after administration of digoxin; this group served as control for the haemodynamic parameters. Further, in two dogs (Group 3) without previous long-term digitalization and in three dogs (Group 4) pretreated with digoxin as previously described transmural biopsies were taken from the left ventricular wall 10 min after an intravenous dose of digoxin. Group 5 comprised three chronically digitalized dogs from which transmural biopsies were taken 90 min after administration of digoxin. All the dogs received a normal diet and no medication apart from digoxin was given. Four of the animals, those with the highest plasma concentrations of digoxin, lost their appetite after five to six days of digitalization, but no vomiting or diarrhoea was observed. No electrocardiographic sign of digitalis toxicity was found.

### *Experimental procedure*

Anaesthesia was induced with narcobarbitone (Narcodorm) and following tubocurarine and pethidine, orotracheal intubation was performed and a respirator connected. Anaesthesia and relaxation were maintained with pethidine, tubocurarine, and $N_2O/O_2$, 3 + 1.5 litres/min in a to-and-fro system.

Left-sided thoracotomy was performed and the left atrium was catheterized through the left auricle. Retrograde catheterization of the left ventricle was performed from the left femoral artery. In three dogs the aorta was catheterized instead of the left atrium. Atrial and left ventricular or aortic pressures were measured by Elema Schönander transducers (type EMT 35). The pressures and a standard limb lead of the ECG were recorded simultaneously. The first derivative of the ventricular pressure curve, i.e. left ventricular $dp/dt_{max}$, was obtained by means of an R/C differentiating unit.

When haemodynamic stability was established, pethidine and tubocurarine were discontinued and halothane was added to the anaesthetic mixture in a concentration of about 0.7%, sufficient to reduce the $dp/dt_{max}$ by about 25–50%. Sixty minutes were allowed for stabilization at this level before the intravenous administration of 0.05 mg digoxin/kg body weight. This digoxin dose is known to cause a positive inotropic effect but no toxic symptoms in dogs without previous long-term digitalization (Steiness & Valentin, 1976).

### *Blood samples*

Heparinized arterial blood was drawn for measurement of plasma concentrations of digoxin, potassium, and sodium just before and 5, 10, 15, 20, 30, 45, 60, 75, and 90 min after the intravenous injection of digoxin. Arterial $Po_2$, $Pco_2$, and pH were measured before and 10, 30, 60, and 90 min after administration of digoxin in Group 1.

### *Biopsies*

In Groups 1 and 2 biopsy specimens weighing 200–400 mg were taken from the left ventricular wall, about one quarter to one third of the walls thickness, 15 min before the intravenous administration of digoxin. In Group 1 myocardial biopsies were again taken after 10, 30, 60, and 90 minutes. In Group 2 an additional biopsy was taken after 90 min only.

Transmural biopsy specimens were taken from two different parts of the left ventricle of the animals in Groups 3, 4, and 5. Each of these specimens was divided into three layers of the wall, i.e. the subendocardial, the mesocardial, and the subepicardial.

Any blood loss, including sampling, was replaced with an electrolyte containing plasma expander (Haemaccel), and water loss with isotonic glucose.

All animals were killed at the end of the experiment.

### *Analytical procedure*

Plasma and myocardial concentrations of digoxin were measured in duplicate by radioimmunoassay as previously described (Steiness, 1974; Steiness & Valentin, 1976). The sensitivity of the method is 0.25 nmol/l plasma and 10 nmol/kg tissue; the precision is 0.06 nmol/l plasma and 5 nmol/kg tissue. The small size of the single myocardial biopsy specimens did not allow measurements of digoxin in subcellular fractions.

Plasma potassium and sodium were determined in duplicate by flame-photometry. Tissue potassium was determined in triplicate according to Valentin & Olesen (1973) and expressed in mmol/kg fat-free solids.

Blood gases and pH were measured with a Radiometer.

### *Statistics*

All the statistical tests used were non-parametric and based on the ranking of results. The Wilcoxon test for paired data was used for the determination of statistical significance. For comparison between different groups, the Wilcoxon test for non-paired data was used. When more than two sets of data from the same group were compared, the Friedman two-way analysis of variance was used (for methods see Geigy Scientific Tables, 1970).

## Results

As shown in Table 1 there was no correlation between the digoxin concentration in the plasma and in the myocardium at steady state.

After the intravenous administration of digoxin, a rapid increase of plasma digoxin concentration was followed by a gradual decrease (Figure 1). The myocardial digoxin concentration increased sigficantly and 10 min after the injection it was four to five times higher than the steady state level ($P < 0.01$) (Table 1, Group 1). Later the myocardial digoxin concentration decreased, and after 30 min it was significantly lower than the 10 min value ($P < 0.02$) but still significantly higher than the initial level ($P < 0.01$). This decrease was followed by a secondary increase ($P < 0.02$) and the 10 min level was reached about 60 min after the digoxin administration. Between 60 and 90 min a minor decrease in myocardial digoxin concentration was observed. The myocardial digoxin concentrations before and 90 min after digoxin administration in Group 2 were within the ranges of those observed in Group 1 (Table 1).

In dogs without previous long-term digitalization the digoxin concentrations found in the subepicardial, subendocardial and mesocardial layers 10 min after intravenous administration of 0.05 mg digoxin/kg body weight did not differ significantly from one another (Table 2, Group 3). Similar results were observed 90 min after digoxin administration in the chronically digitalized dogs (Table 2, Group 5). However, in the chronically digitalized dogs the digoxin concentrations found in the mesocardial layers of the myocardium after 10 min were significantly higher than in the other layers ($P < 0.05$).

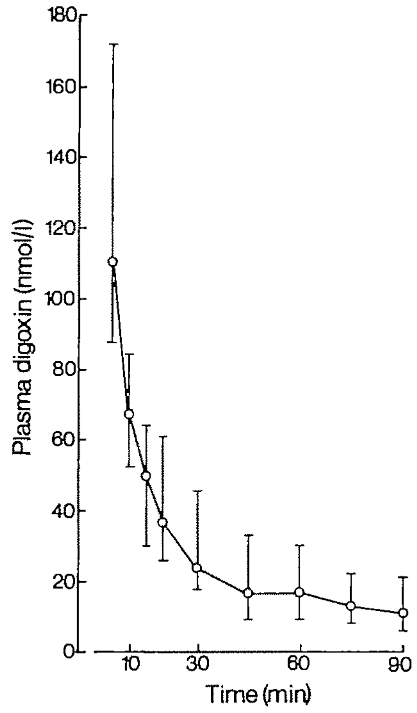


**Figure 1** Median plasma digoxin concentrations after intravenous administration of 0.05 mg digoxin/kg body weight to chronically digitalized dogs.

**Table 1** Steady state plasma digoxin concentrations and myocardial concentrations of digoxin before and after intravenous administration of digoxin to chronically digitalized dogs. (Figures in parentheses are µg/l plasma or µg/kg tissue)

| | *Dog no.* | *Plasma digoxin in nmol/l (or µg/l)* | *Myocardial digoxin in nmol/kg (or µg/kg) Minutes after digoxin* | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 | 10 | 30 | 60 | 90 |
| Group 1 | 1 | 1.4 (1.1) | 56 (44) | 189 (148) | 159 (125) | 230 (180) | 218 (171) |
| | 2 | 1.2 (0.9) | 78 (61) | 302 (237) | 238 (187) | — | 206 (162) |
| | 3 | 0.6 (0.5) | 149 (117) | 509 (399) | 337 (264) | 570 (447) | 457 (358) |
| | 4 | 1.8 (1.4) | 13 (10) | 310 (243) | 262 (205) | 300 (235) | 202 (158) |
| | 5 | 2.3 (1.8) | 40 (31) | 134 (105) | 164 (129) | 158 (124) | 187 (147) |
| | 6 | 0.8 (0.6) | 33 (26) | 216 (169) | 103 (80) | 175 (137) | 245 (192) |
| | 7 | 0.6 (0.5) | 137 (107) | 375 (294) | 302 (237) | 384 (301) | 321 (252) |
| | 8 | 1.4 (1.1) | 66 (52) | 430 (337) | 379 (297) | 429 (336) | 349 (274) |
| | Median | 1.3 (1.0) | 60 (47) | 306 (245) | 250 (196) | 300 (235) | 232 (182) |
| Group 2 | 9 | 2.6 (2.0) | 96 (75) | — | — | — | 323 (253) |
| | 10 | 1.7 (1.3) | 49 (38) | — | — | — | 244 (191) |
| | 11 | 2.3 (1.8) | 135 (106) | — | — | — | 358 (281) |

### *Electrolytes*

Only minor changes in plasma concentrations of potassium and sodium were observed. The myocardial concentration of potassium decreased rapidly after digoxin administration (Table 3, Group 1) and after 30 min was significantly lower than the initial content ($P < 0.02$). The decrease of the myocardial potassium concentration continued, although not significantly, during the remaining part of the study.

### *Haemodynamics*

The contractility, $dp/dt_{max}$, increased within a few minutes after the administration of digoxin. Ten minutes after the administration, $dp/dt_{max}$ had increased by a mean of 35% (Table 4). After repeated biopsy sampling in Group 1 some reduction in $dp/dt_{max}$ was seen. By contrast, the control animals of Group 2 which were biopsied only at 0 and 90 min maintained steady $dp/dt_{max}$ (Table 4). Other

**Table 2** The transmural myocardial content of digoxin (nmol/kg tissue and in parentheses µg/kg tissue) 10 or 90 min after an intravenous dose of 0.05 mg digoxin/kg body weight

| *Treatment* | *Group* | *10 min after digoxin* | | | *90 min after digoxin* | | |
|---|---|---|---|---|---|---|---|
| | | *Subepicardial* | *Mesocardial* | *Subendocardial* | *Subepicardial* | *Mesocardial* | *Subendocardial* |
| No long-term digitalization | 3 | 289 | 265 | 322 | — | — | — |
| | | 322 | 338 | 321 | — | — | — |
| | | 260 | 242 | 275 | — | — | — |
| | | 297 | 310 | 335 | — | — | — |
| | Median | 293(229) | 276(216) | 322(252) | | | |
| Chronic digitalization | 4 | 313 | 378 | 312 | — | — | — |
| | | 483 | 710 | 600 | — | — | — |
| | | 656 | 750 | 550 | — | — | — |
| | | 362 | 486 | 403 | — | — | — |
| | | 439 | 562 | 467 | — | — | — |
| | Median | 439(344)* | 562(441) | 467(366)* | | | |
| Chronic digitalization | 5 | — | — | — | 439 | 455 | 486 |
| | | — | — | — | 420 | 443 | 467 |
| | | — | — | — | 151 | 184 | 135 |
| | | — | — | — | 170 | 169 | 184 |
| | | — | — | — | 512 | 493 | 580 |
| | Median | | | | 420(329) | 443(347) | 467(366) |

* $P < 0.05$.

**Table 3** Myocardial concentration of potassium (mol/kg fat free tissue) before and after an intravenous digoxin dose given to chronically digitalized dogs ($n = 8$)

| | *Minutes after digoxin* | | | | |
|---|---|---|---|---|---|
| | 0 | 10 | 30 | 60 | 90 |
| Median | 369 | 354 | 350* | 348* | 328* |
| Range | 336–381 | 291–390 | 299–378 | 310–379 | 301–377 |

* $P < 0.02$; ** $P < 0.01$.

**Table 4** Left ventricular contractility ($dp/dt_{max}$) before and after intravenous administration of digoxin 0.05 mg/kg body weight to chronically digitalized dogs (% of initial value)

| | | | *Minutes after digoxin* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Group* | | | 0 | 5 | 10 | 15 | 20 | 30 | 45 | 60 | 75 | 90 |
| 1<br>($n=8$) | $dp/dt_{max}$<br>% change | Median | 100 | 117** | 135** | 115 | 110 | 120 | 120 | 118 | 118 | 118 |
| | | Range | | 5–55 | 5–100 | 0–75 | 0–90 | 5–90 | 0–110 | 0–100 | 0–100 | 0–100 |
| | $dp/dt_{max}$ | Dog 9 | 100 | 119 | 120 | 122 | 125 | 125 | 119 | 125 | 125 | 130 |
| 2 | $dp/dt_{max}$ | Dog 10 | 100 | 130 | 130 | 130 | 140 | 140 | 140 | 140 | 150 | 140 |
| | $dp/dt_{max}$ | Dog 11 | 100 | 120 | 120 | 125 | 124 | 130 | 130 | 130 | 124 | — |

** $P<0.01$.

haemodynamic parameters, left ventricular systolic and end-diastolic pressures and ventricular rate, remained unchanged, and the ECG demonstrated sinus rhythm during the study. No change in pH, $P\text{O}_2$, and $P\text{CO}_2$ was observed.

## Discussion

Since it became possible to measure plasma concentrations of digoxin (Smith, Butler & Haber, 1969), many investigations have revealed fairly good correlation between the steady state digoxin concentration in plasma and its therapeutic effect (Chamberlain, White, Howard & Smith, 1970; Beller, Smith, Abelmann, Haber & Hood, 1971; Evered & Chapman, 1971; Bertler, Gustafson, Ohlin, Monti & Redfors, 1973). Therefore, it has been argued that the plasma digoxin concentration is related to the myocardial digoxin concentration. Indeed Coltart, Howard & Chamberlain (1972) and Redfors, Bertler & Schuller (1973) found correlation between the concentration of digoxin in plasma and atrial tissue in man. On the other hand, it has been difficult to demonstrate a similar correlation between the digitalis concentration in plasma and in the ventricle, possibly because of a varying degree of fibrosis in the human material (Storstein, 1973; Coltart, Gullner, Billingham, Goldman, Stinson, Kalman & Harrison, 1974). Nevertheless, no correlation between the plasma and the left ventricular concentration of digoxin was found in the present study of non-fibrotic dog hearts (Table 1).

Recent studies suggested the existence of at least two different myocardial receptor sites for digoxin (Okita *et al.*, 1973; Steiness & Valentin, 1976). The supposition is made that by measuring the decrease in myocardial potassium content as a term of the inhibition of the Na-K APTase, the binding of digoxin to one of these kind of receptors can be estimated from the effect of the binding. Binding to the 'inotropic' receptor might similarly be estimated from the development of the positive inotropic effect ($dp/dt_{max}$) (Klaus *et al.*, 1961; Grupp & Charles, 1964; Okita, Richardson, Roth-Schechter & Thomas, 1969; Roth-Schechter, Okita, Thomas & Richardson, 1970; Lüllmann & Ravens, 1973; Okita *et al.*, 1973; Steiness & Valentin, 1976). It was not possible, however, to evaluate the digoxin bound to the 'inotropic' receptor at steady state. Our technique for measurement of contractility ($dp/dt_{max}$) did not permit comparison between the absolute values of $dp/dt_{max}$ from one series to another, but allowed only the following of changes of $dp/dt_{max}$ in the same animal during the study.

The myocardial uptake of digoxin after intravenous digoxin in dogs without long-term digitalization was recently demonstrated to be very fast initially and to

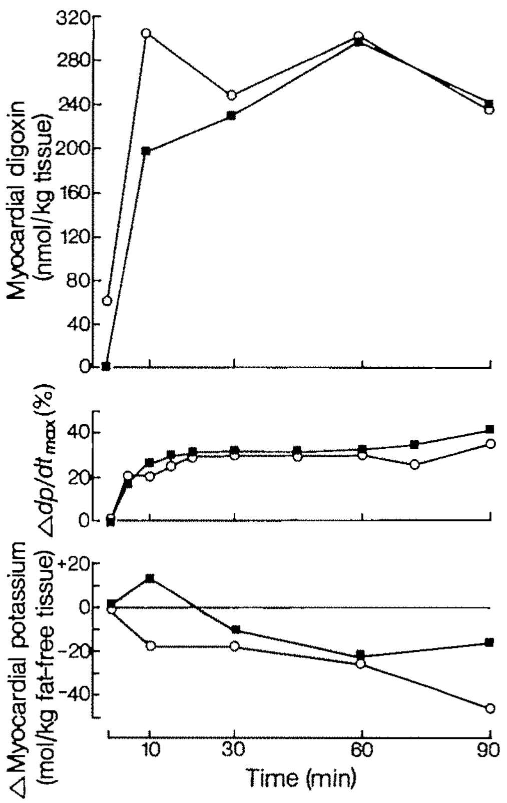


**Figure 2** The myocardial concentrations of digoxin (median), the inotropic effect (median), and the myocardial potassium content (median) at intervals after an intravenous digoxin dose (0.05 mg/kg body weight) in chronically digitalized dogs (○) and in dogs without previous long-term digitalization (■); (Steiness & Valentin, 1976).

continue for 60 min (Figure 2 and Steiness & Valentin, 1976). Chronically digitalized dogs also had a very rapid initial uptake of digoxin in the myocardium but the subsequent shape of the uptake curves differed significantly (Figure 2). This difference could be due to a change of the digoxin affinity for one or both of the digoxin receptor sites. However, the positive inotropic effect, which is postulated to express the amount of digoxin bound to the 'inotropic' receptor, was quite similar to that produced by the same intravenous dose of digoxin in dogs without previous long-term digitalization (Figure 2). If this receptor had a changed affinity for digoxin, a parallel change in the inotropic response would be expected. It is therefore suggested that the 'inotropic' receptor has an unchanged affinity for digoxin after long-term digitalization with the doses given in the present investigation.

The affinity of digoxin to the Na-K APTase correlated receptor was also unchanged. As seen from Figure 2 the loss of myocardial potassium per unit time in the chronically digitalized dogs was equal to that in non-digitalized dogs, except during the first 10 minutes.

The loss of myocardial potassium following intravenous digoxin in non-digitalized dogs begins after about 15 min (Steiness & Valentin, 1976). This was interpreted as a slow binding of digoxin to the Na-K ATPase correlated receptor. In chronically digitalized dogs, however, the myocardial content of potassium under steady state conditions was not appreciably lower than in dogs without previous long-term digitalization (Steiness & Valentin, 1976). An additional increase of the digoxin receptor concentration must, therefore, produce further inhibition of the ATPase with a decrease of the myocardial content of potassium within 10 minutes.

The significant change in myocardial uptake of digoxin (Table 1 and Figure 2) may therefore be due to changes in the myocardial distribution of non-specifically bound digoxin rather than to changes in the digoxin uptake by one of the specific receptor sites. The myocardial biopsies in Group 1 were taken from the surface of the ventricular wall and were about one quarter to one third of the walls thickness. The biopsy involved the subepicardial layer and different amounts of the mesocardial layer of the ventricular wall. To elucidate the hypothesis that the myocardial distribution of digoxin is changed in chronically digitalized dogs, the transmural distribution of digoxin in the myocardium was investigated. Uniform concentrations of digoxin were found in different layers of the left ventricular wall 10 min after the intravenous administration of digoxin to non-digitalized dogs. On the other hand, in the chronically digitalized dogs both the subendocardial and the subepicardial concentrations were found to be significantly lower than the mesocardial concentration at 10 min but not at 90 min after digoxin administration. The difference between the non-uniform distribution of digoxin in chronically digitalized dogs and the uniform distribution in dogs without previous long-term digitalization suggests an initial slower rate of digoxin uptake into the subepicardial and the subendocardial layer in chronically digitalized dogs, that could be due to a change in myocardial blood flow. Our results do not permit evaluation of this suggestion.

This work was supported by grants from Fonden til Laegevidenskabens Fremme, Statens Laegevidenskabelige Forskningsråd and Hjerteforeningen.

## References

BELLER, G.A., SMITH, T.W., ABELMANN, W.H., HABER, E. & HOOD, W.B. (1971). Digitalis intoxication. *New Engl. J. Med.*, **284**, 989–997.

BERTLER, A.A., GUSTAFSON, A., OHLIN, P., MONTI, M. & REDFORS, A. (1973) Digoxin intoxication and plasma glycoside levels. *Symposium on Digitalis*, p. 300, Gyldendal Norsk Forlag.

CHAMBERLAIN, D.A., WHITE, R.J., HOWARD, M.R. & SMITH, T.W. (1970). Plasma digoxin concentrations in patients with atrial fibrillation. *Br. med. J.*, **3**, 429–432.

COLTART, J., HOWARD, M. & CHAMBERLAIN, D. (1972). Myocardial and skeletal muscle concentrations of digoxin in patients on long-term therapy. *Br. med. J.*, **2**, 318–319.

COLTART, J., GULLNER, H.G., BILLINGHAM, M., GOLDMAN, R.H., STINSON, E.B., KALMAN, S.M. & HARRISON, D.C. (1974). Physiological distribution of digoxin in human heart. *Br. Heart J.*, **4**, 733–736.

DEUTSCHER, R.N., HARRISON, D.C. & GOLDMAN, R.H. (1972). The relation between myocardial $^3$H-digoxin concentration and its hemodynamic effect. *Am. J. Cardiol.*, **29**, 47–55.

DOHERTY, J.E. & PERKINS, W.H. (1966). Tissue concentration and turnover of tritiated digoxin in dogs. *Am. J. Cardiol.*, **17**, 47–58.

EVERED, D.C. & CHAPMAN, C. (1971). Plasma digoxin concentrations and digoxin toxicity in hospital patients. *Br. Heart J.*, **33**, 540–545.

GEIGY SCIENTIFIC TABLES (1970). ed. Diem, K. & Lentner, C. Basel: J. R. Geigy, S.A.

GOLDMAN, R.M., DEUTSCHER, R.H., SCHWEIZER, E. & HARRISON, D.C. (1972). Effect of a pharmacologic dose of digoxin on inotropy in hyper- and normokalemic dogs. *Am. J. Physiol.*, **223**, 1438–1444.

GRUPP, G. & CHARLES, A. (1964). Effect of ouabain and 3-acetylstrophanthidin on potassium exchange in the dog heart in situ. *J. Pharmac. exp. Ther.*, **143**, 356–365.

KLAUS, W., KUSCHINSKY, G. & LÜLLMANN, F. (1961). The influence of therapeutic and toxic concentrations of digitoxigenin on the K-flux and the ion concentrations in isolated guinea pig atria. *Biochem. Pharmac.*, **8**, 37–63.

LANGER, G.A. (1972). Effects of digitalis on myocardial ionic exchange. *Circulation*, **46**, 180–187.

LÜLLMANN, H. & RAVENS, U. (1973). The time course of the changes in contractile force and in transmembrane potentials induced by cardiac glycosides in guinea-pig papillary muscle. *Br. J. Pharmac.*, **49**, 377–390.

OKITA, G.T., RICHARDSON, F.F., ROTH-SCHECHTER, B.F. & THOMAS, R.E. (1969). Indirect evidence for the dissociation of Na$^-$, K$^+$-activated ATPase inhibition from positive inotropic action of strophanthidin-3-bromacetate. *Fedn. Proc.*, **28**, 607.

OKITA, G.T., RICHARDSON, F.F. & ROTH-SCHECHTER, B.F. (1973). Dissociation of the positive inotropic action of digitalis from inhibition of sodium- and potassium-activated adenosine triphosphatase. *J. Pharmac. exp. Ther.*, **185**, 1–11.

REDFORS, A., BERTLER, A.A. & SCHULLER, H. (1973). The ratio between myocardial and plasma levels of digoxin in man. *Symposium on Digitalis*, p. 265, Gyldendal Norsk Forlag.

ROTH-SCHECHTER, B.F., OKITA, G.T., THOMAS, R.E. & RICHARDSON, F.F. (1970). On the positive inotropic action of alkylating bromacetates of strophanthidin and strophantidol-(19-H$^3$). *J. Pharmac. exp. Ther.*, **171**, 13–19.

SCHWARTZ, A., MATSUI, H. & LAUGHTER, A.H. (1968). Tritiated digoxin binding to (Na$^+$-K$^+$)-activated adenosine triphosphatase: Possible allosteric site. *Science*, **160**, 323–325.

SMITH, T.W., BUTLER, V.P. & HABER, E. (1969). Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay. *New Engl. J. Med.*, **281**, 1212–1217.

STEINESS, E. (1974). Renal tubular secretion of digoxin. *Circulation*, **50**, 103–109.

STEINESS, E. & VALENTIN, N. (1976). Myocardial digoxin uptake. Dissociation between digitalis induced inotropism and myocardial loss of potassium. *Br. J. Pharmac.* (in press).

STORSTEIN, L. (1973). The ratio between myocardial and serum levels of digitoxin. *Symposium on Digitalis*, p. 249, Gyldendal Norsk Forlag.

VALENTIN, N. & OLSEN, K.H. (1973). Measurement of muscle tissue water and electrolytes. *Scand. J. clin. lab. Invest.*, **32**, 155–161.

*(Received March 22, 1976.*
*Revised June 3, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 561–567

# STUDIES ON THE MECHANISM OF ACTION OF AMANTADINE

F. BROWN & P.H. REDFERN

School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY

1 The effect of amantadine hydrochloride on various aspects of catecholamine metabolism in the rat brain has been investigated.

2 Amantadine failed to have any significant effect on brain concentrations of dopamine or noradrenaline even when administered daily for 9 days.

3 Amantadine had no effect on the rate of decline of noradrenaline and dopamine concentrations after $\alpha$-methyl-$p$-tyrosine.

4 *In vitro*, amantadine inhibited dopamine uptake into synaptosomes only at high concentrations, and caused little release of dopamine from synaptosomes.

5 There is no evidence from these results to suggest that the anti-Parkinsonian effect of amantadine is related to an action on dopaminergic mechanisms.

## Introduction

It is generally believed that the anti-Parkinsonian drug amantadine owes its effectiveness to a central action on dopaminergic mechanisms. However, there is a lack of concensus as to the exact mechanism of action of the drug. Direct stimulation of dopamine receptors (Papeschi, 1974), blockade of neuronal uptake (Fletcher & Redfern, 1970; Heimans, Rand & Fennessy, 1972; Baldessarini, Lipinski & Chace, 1972), catecholamine release (Scatton, Cheramy, Besson & Glowinski, 1970; Von Voigtlander & Moore, 1971; Stromberg & Svensson, 1971; Farnebo, Fuxe, Goldstein, Hamberger & Ungerstedt, 1971) and increased synthesis (Scatton *et al.*, 1970) have all been suggested to be responsible. We have investigated this problem by examining the effect of amantadine on brain concentrations of catecholamines, on the turnover of these amines in the brain, and on the uptake and release of dopamine from isolated synaptosome preparations. A preliminary report of these results was presented to the British Pharmaceutical Conference (Brown & Redfern, 1974).

## Methods

### *Estimation of noradrenaline and dopamine concentrations*

Groups of 10 male Sprague–Dawley rats weighing 150 to 250 g received either 0.9% w/v NaCl solution (saline) or amantadine intraperitoneally and were killed 0.5, 1 or 2 h after injection. The brains were quickly removed and homogenized in 8 ml of 0.4N $HClO_4$ containing 0.1% $Na_2S_2O_5$ and 0.05% disodium edetate. Homogenates were washed into ulta-centrifuge tubes with a further 1 ml of $HClO_4$ and, after centrifuging at 25,000 *g* for 15 min, the clear supernatant was carefully decanted. The noradrenaline and dopamine concentrations in 6 ml aliquots of the supernatants were assayed fluorimetrically after isolation on alumina (Shellenberger & Gordon, 1971).

### *Estimation of noradrenaline and dopamine turnover*

Groups of male Sprague–Dawley rats were injected intraperitoneally with 250 mg/kg $\alpha$-methyl-$p$-tyrosine methyl ester hydrochloride (AMPT) and brain concentrations of noradrenaline and dopamine were estimated by the method described above in different groups of animals at intervals after injection. The rate of decline of dopamine and noradrenaline levels was calculated by log linear regression analysis of the results obtained during the 4 h after injection.

### *Dopamine uptake into brain homogenates*

The method was based on that of Snyder & Coyle (1969). Male Sprague–Dawley rats were killed by decapitation and the brains quickly removed and placed on ice. Whole brains or discrete brain areas were homogenized in 0.32M sucrose adjusted to pH 7.0 with sodium bicarbonate, using a teflon-in-glass homogenizer with 0.254 mm clearance, and the homogenates were centrifuged at 4 °C at 1000 *g* for 10 minutes. Aliquots (0.5 ml) of the synaptosome-rich supernatant were incubated with 4.5 ml of modified Krebs solution for 5 min at 37 °C. After this period of equilibration, radioactively-labelled dopamine was added. The labelled dopamine bound by the synaptosomes was then recovered at various times using one of two methods: (i) 5.0 ml of incubation medium at 0–4 °C was added to the incubation mixture and the tubes were centrifuged at 50,000 *g* for 20 minutes. The pellets were collected, washed and dispersed in 0.5 ml of 0.5 M NaOH. Aliquots of the digested pellets were taken for liquid scintillation counting in 'Unisolve' (Koch-Light). (ii) 0.5 ml of the incubation mixture was added to 5.0 ml of cold incubation medium and this mixture was quickly passed through a millipore membrane (0.22 μm pore size). After washing with a further 5.0 ml of cold medium, the filter disc was dried and placed in a scintillation vial. The radioactive content of the dried membrane was measured by liquid scintillation counting after shaking with 0.5 ml distilled water and adding 9.5 ml 'Unisolve'.

### *Dopamine release from synaptosomes*

The method was essentially the same as that used in the uptake experiments using millipore filters for rapid separation of the synaptosomes from the incubating medium. After a 5 min incubation the radioactive dopamine content of the synaptosomes reached a maximum. A significant decline in radioactive content

**Table 1** The effect of amantadine on dopamine and noradrenaline concentrations in rat brain

*Dopamine (ng/g ± s.e. mean)*

| *Time after amantadine (h)* | *Dose of amantadine (mg/kg i.p.)* | | | | | | *No. of rats per group* |
|---|---|---|---|---|---|---|---|
| | 0 | 10 | 40 | 80 | 160 | 94 mg/kg per day | |
| 0.5 | 787 ∓ 23 | | | 791 ∓ 12 | | | 10, 10 |
| 1.0 | 835 ∓ 21 | 806 ∓ 21 | | | | | 10, 10 |
| | 907 ∓ 32 | | 932 ∓ 15 | | | | 10, 10 |
| | 811 ∓ 17 | | | 790 ∓ 16 | | | 10, 10 |
| | 866 ∓ 24 | | | 788 ∓ 18* | | | 10, 10 |
| | 954 ∓ 43 | | | | 958 ∓ 49 | | 8, 7 |
| 2.0 | 893 ∓ 43 | 979 ∓ 29 | | | | | 6, 6 |
| | 776 ∓ 22 | | | 781 ∓ 28 | | | 9, 9 |
| 3.0 | 712 ∓ 36 | | 714 ∓ 24 | 739 ∓ 42 | | | 9, 8, 8 |
| Chronic treatment | 838 ∓ 12 | | | | | 774 ∓ 30 | 10, 10 |

*Noradrenaline (ng/g ∓ s.e. mean)*

| *Time after amantadine (h)* | *Dose of amantadine (mg/kg i.p.)* | | | | | | *No. of rats per group* |
|---|---|---|---|---|---|---|---|
| | 0 | 10 | 40 | 80 | 160 | 94 mg/kg per day | |
| *0.5* | 443 ∓ 22 | | | 476 ∓ 23 | | | 10, 10 |
| *1.0* | 412 ∓ 18 | 402 ∓ 20 | | | | | 10, 10 |
| | 533 ∓ 23 | | 593 ∓ 18 | | | | 10, 10 |
| | 408 ∓ 13 | | | 456 ∓ 14* | | | 10, 10 |
| | 432 ∓ 13 | | | 430 ∓ 13 | | | 10, 10 |
| | 416 ∓ 8 | | | | 402 ∓ 19 | | 8, 7 |
| *2.0* | 480 ∓ 16 | 490 ∓ 11 | | | | | 6, 6 |
| | 441 ∓ 21 | | | 463 ∓ 30 | | | 9, 9 |
| *3.0* | 422 ∓ 31 | | 405 ∓ 25 | 352 ∓ 32 | | | 9, 8, 8 |
| *Chronic treatment* | 421 ∓ 10 | | | | | 430 ∓ 16 | 10, 10 |

*$P < 0.05$

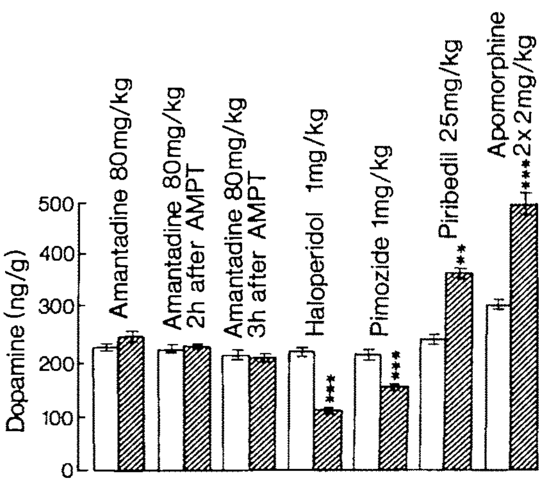


**Figure 1** Dopamine concentrations in rat brain 4 h after $\alpha$-methyl-$p$-tyrosine (AMPT, 250 mg/kg i.p.). Open columns=AMPT alone; hatched columns =AMPT+drug. Vertical bars show s.e. means. Drugs were generally administered at the same time as AMPT. Apomorphine was given in two doses, one with the AMPT and one 2 h later.
Differences from corresponding controls: $^{**}P<0.01$; $^{***}P<0.001$.

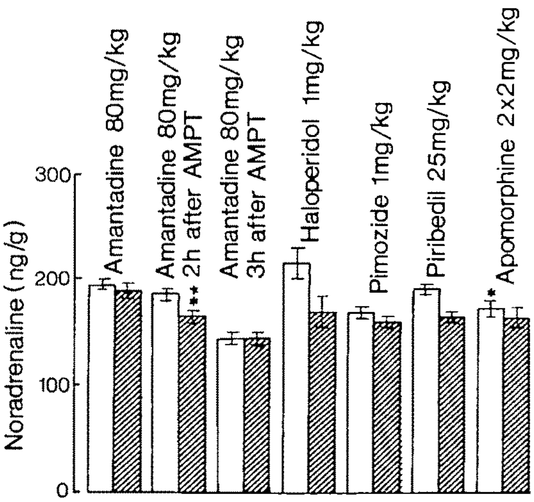


**Figure 2** Noradrenaline concentrations in rat brain 4 h after $\alpha$-methyl-$p$-tyrosine (AMPT 250 mg/kg i.p.). Open columns=AMPT alone; hatched columns =AMPT+drug. Vertical bars show s.e. means. The times of drug administration were the same as in Figure 1.
Differences from corresponding controls: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

following the addition of drugs to the incubation mixture at this point was taken to indicate release of previously-bound radioactive dopamine from the synaptosomes.

### *Drugs*

The following drugs were donated: amantadine hydrochloride (Geigy Pharmaceuticals Ltd.), rimantadine hydrochloride (E.T. Du Pont de Nemours and Co. Ltd.), piribedil methane sulphonate (Servier Laboratories Ltd.), apomorphine hydrochloride (Macfarlan Smith Ltd.), haloperidol (G.D. Searle and Co. Ltd.) and pimozide (Janssen Pharmaceuticals). (+)-Amphetamine sulphate and $\alpha$-methyl-$p$-tyrosine methylester hydrochloride were obtained from Sigma London Chemical Co. Ltd.

## Results

Table 1 shows the concentrations of noradrenaline and dopamine in the rat brain after various doses of amantadine. It can be seen that in only one experiment was there any significant change in amine levels. After 80 mg/kg amantadine, dopamine concentrations were decreased while noradrenaline concentrations were increased. Both these changes were just significant at the 95% level, and were not seen when the experiment was repeated. Even when rats were treated for 9 days with amantadine (administered in the drinking water in an approximate daily dose of 94 mg/kg) dopamine and noradrenaline levels were not significantly altered.

Figures 1 and 2 respectively show the effect of drugs on the rate of decline of dopamine and noradrenaline concentrations after AMPT. It can be seen that drugs which are believed to exert a direct action on dopamine receptors had a significant effect on dopamine turnover. For instance the dopamine antagonist pimozide, 1 mg/kg, injected 30 min before AMPT significantly increased the rate of decline of dopamine concentrations ($P<0.001$), while there was no change in noradrenaline concentrations. Haloperidol, 1 mg/kg, had a similar effect. Conversely when dopamine receptor stimulants such as piribedil 25 mg/kg and apomorphine 2 x 2 mg/kg were administered, the consequent inhibition of dopamine release produced an equally significant reduction in the rate of decline in dopamine concentration.

As can be seen from Figure 1, amantadine (80 mg/kg i.p.) produced no measurable change in dopamine turnover whether administered simultaneously with the AMPT or 2 h or 3 h afterwards. A slight but statistically significant ($P<0.01$) fall in noradrenaline levels was observed when amantadine (80 mg/kg) was administered 2 h after AMPT, but not when the same dose was given either at the same time as the AMPT or 3 h afterwards (Figure 2).

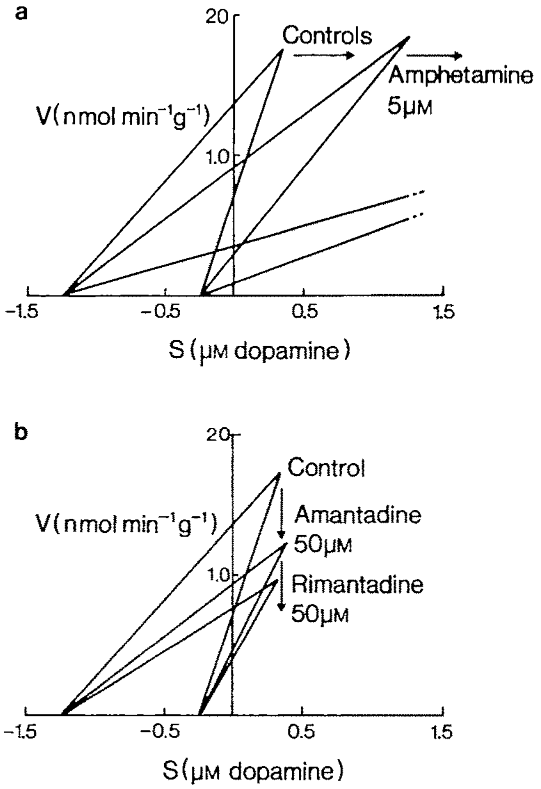


**Figure 3** Direct linear plots (Eisenthal & Cornish-Bowden, 1974) of dopamine uptake into synaptosomes prepared from rat corpus striatum: (a) competitive inhibition produced by (+)-amphetamine; (b) non-competitive inhibition produced by amantadine. Each line is the mean of 3 separate determinations at one substrate concentration.

*Effects of amantadine* in vitro

The effect of amantadine on dopamine uptake into synaptosomes, displayed according to the method of Eisentahl & Cornish-Bowden (1974) is compared to that of (+)-amphetamine and of rimatadine in Figure 3. Both amantadine and rimantadine, a close analogue of amantadine lacking clinically useful anti-Parkinsonian properties, are shown to be relatively weak noncompetitive inhibitors of dopamine uptake, with *Ki* values of 125 μM and 69.9 μM respectively. Amphetamine was a relatively potent competitive inhibitor, with a *Ki* of 1.94 μM.

In spite of considerable differences in control figures for dopamine uptake in different brain regions, especially between those of the striatum and other areas, amantadine caused a similar inhibitory effect, relative to controls, in all areas. Amantadine also failed to show any selective effect against dopamine uptake compared to uptake of either noradrenaline or 5-hydroxytryptamine. Finally, pretreatment of rats with amantadine (50 mg/kg i.p.) 1 h before preparation of homogenates did not result in any significant inhibition of dopamine uptake as compared to saline pretreated controls.

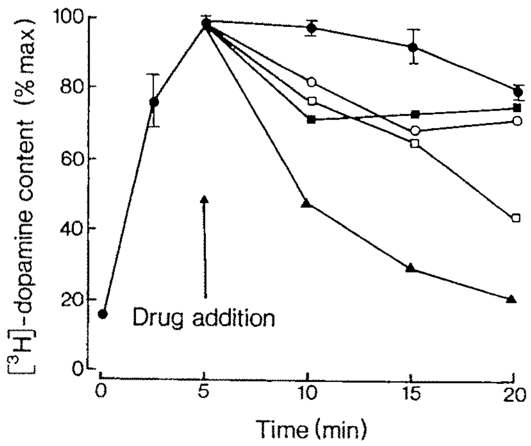


**Figure 4** Effect of drugs on release of radioactive dopamine from synaptosomes prepared from rat corpus striatum: (●) control; (■) amantadine 100 μM; (○) piribedil 100 μM; (□) ouabain 100 μM; (▲) tyramine 10 μM.

[$^3$H]-dopamine uptake into striatal homogenates reached a maximum after about 5 min incubation. Thereafter there was a gradual decline. Figure 4 shows the effect of adding various drugs to the incubation mixture after 5 min incubation. It can be seen that neither amantadine 100 μM nor piribedil 100 μM produced any marked decline in [$^3$H]-dopamine content, when compared to the effect of tyramine 10 μM. Indeed, the decline in [$^3$H]-dopamine content produced by ouabain 100 μM was more marked than that due to amantadine or piribedil.

## Discussion

Despite the numerous reports of the clinical efficacy of amantadine in Parkinson's disease, the mode of action of the drug remains unclear. Since the drug has minimal antimuscarinic properties (Vernier, Harman, Stump, Lynes, Marvel & Smith, 1969), some action on dopaminergic mechanisms seems most likely to account for its clinical action, yet no consistent evidence as to the exact nature of this action exists. For instance, various workers have used a variety of methods to estimate brain amines in the rat after amantadine treatment. Baldessarini *et al.* (1972) reported a fall in dopamine levels, while Stromberg & Svensson (1971), Abuzzahab (1971), Bak, Hassler, Kim & Kataoka (1972), Maj, Sowinska & Baran (1972) and Papeschi (1974) all reported unchanged dopamine levels. Similarly while Baldessarini *et al.* (1972) and Stromberg & Svensson (1971) found decreased concentrations of noradrenaline after amantadine, Abuzzahab (1971), Maj *et al.* (1972) and Papeschi (1974) found no change. In our experiments, despite the fact that both the time scale and dose range studied included those at which maximal behavioural effects occur (Stromberg & Svensson, 1971; Maj *et al.*, 1972; Davies, Jackson & Redfern, 1973) no significant changes in noradrenaline or dopamine concentrations were obtained even after chronic treatment.

In the experiments with AMPT, it is evident that drugs which stimulate dopamine receptors, such as apomorphine (Ernst, 1967) or piribedil (Costall & Naylor, 1973) caused slowing of dopamine turnover, while receptor blocking drugs like haloperidol and pimozide (Janssen, 1967, Anden, Butcher, Corrodi, Fuxe & Ungerstedt, 1970) caused an acceleration of turnover. The simplest interpretation of these results is feedback inhibition or activation of dopaminergic neurones, presumably in an attempt to maintain homeostasis, because the changes in turnover are dependent on nerve impulse activity (Anden, Fuxe & Hokfelt, 1966).

The failure of amantadine to modify dopamine turnover under these circumstances, when compared to the effectiveness of the dopamine receptor stimulants and blockers indicates that amantadine caused no significant activation of postsynaptic dopamine receptors. Such an action has been proposed by Papeschi (1974) who found a higher level of dopamine in rats given amantadine 1 h before AMPT than in animals given only AMPT. However, this effect was only observed at an early stage (1 h) after AMPT administration, when the response is complicated by non-linear decay (Glowinski, 1972).

There are similarly conflicting data from behavioural experiments on the contribution of pre- or postsynaptic events to the action of amantadine. Buus-Lassen (1971; 1973) found that locomotor activity due to amantadine was unaffected by pretreatment with AMPT. These results appear to support a postsynaptic action for amantadine, as do similar results of Maj *et al.* (1972) and Thornburg & Moore (1972). In contrast, results on turning behaviour from unilaterally striectomised animals demonstrate that in this experimental model an indirect or presynaptic action is responsible for the effects of amantadine (Farnebo *et al.*, 1971; Von Voigtlander & Moore, 1973). Since rotational behaviour is more specific for striatal dopaminergic function while both dopamine and noradrenaline may contribute to locomotor responses, we believe the behavioural results are consistent with the results reported here, suggesting a presynaptic action for amantadine. However, the results of our *in vitro* experiments do not provide any clear indication of the exact nature of such an action.

Amantadine was not outstanding as an inhibitor of dopamine uptake into synaptosomes *in vitro*, producing significant inhibition only at high doses (50% inhibition at 100 μM). This finding is supported by results of other workers (Baldessarini *et al.*, 1972; Heikkila & Cohen, 1972; Herblin, 1972) although Heimans *et al.* (1972) reported that amantadine would cause 50% inhibition of dopamine uptake into striatal homogenates at a concentration as low as 3.6 μM.

Direct linear plots of the relationship between the rate of dopamine uptake and the initial dopamine concentration, calculated by the method of Eisenthal & Cornish-Bowden (1974) in which substrate concentration is plotted on the abscissa and the velocity on the ordinate scale, showed that in the presence of amantadine, *Vmax* is displaced vertically downwards, indicating a non-competitive inhibitory effect. In contrast, in the presence of amphetamine the apparent $K_m$ was displaced to the right, indicating competitive inhibition. The fact that amphetamine, a drug generally considered to be a dopamine releasing agent, should apparently be an effective inhibitor of uptake could indicate that the system used is non-specific, i.e. a sudden release of dopamine could give the same result in terms of relative distribution of radioactivity as inhibition of uptake. However, Raiteri, Levi & Federico (1974) showed that, using a superfusion technique, amphetamine was indeed a powerful inhibitor of noradrenaline uptake and had a minimal effect on release. In the *in vitro* system used in these experiments amphetamine, haloperidol, benapryzine and the amantadine analogue, rimantadine, all inhibited dopamine uptake more effectively than did amantadine. This relative ineffectiveness of amantadine, together with the fact that the drug was no more effective in blocking dopamine uptake than in blocking noradrenaline or 5-hydroxytryptamine uptake suggests that this mechanism is unlikely to account for the anti-Parkinsonian action of amantadine.

A dopamine releasing property for amantadine has been claimed by many workers (Stromberg, Svensson & Waldeck, 1970; Stromberg & Svensson, 1971; Farnebo *et al.*, 1971) while others have found it to be a less effective releasing agent than uptake blocking agent (Heikkila & Cohen, 1972; Heimans *et al.*, 1972). As has already been mentioned, it is difficult in an *in vitro* system such as we have used to distinguish between uptake blockade and amine release. Nevertheless comparison of amantadine with other drugs known either to cause dopamine release or to inhibit dopamine uptake provides a reasonable basis for conjecture. On this basis, since a significant decline in dopamine concentration was caused by tyramine, whereas the response to amantadine was less marked even than that to ouabain, it is concluded that amantadine has negligible ability to release dopamine from nerve endings, at least *in vitro*.

The results reported, in common with those of many other workers, appear to show that if amantadine does have an effect on central dopaminergic mechanisms, the effect is a slight one and is certainly not likely to be evident at clinically effective dose levels. Two suggestions can be made from these results. First it is possible that the anti-Parkinsonian activity of amantadine is due to mechanisms unrelated to brain dopamine. Certainly, Bak *et al.* (1972) have shown an increase in striatal acetylcholine and γ-aminobutyric acid concentrations in rat, although high doses (100 mg/kg) were used, while in man, amantadine has been shown to cause an increase in the excretion of 1,4-methylhistamine (Jones, Turnbull, Lenman & Robertson, 1972). The second possibility is that the effect of amantadine on brain dopaminergic mechanisms only becomes evident under the conditions obtaining in a Parkinsonian patient. For instance it has been reported that the increase in excretion of dopamine metabolites produced by amantadine in Parkinsonian patients is not reproduced in non-Parkinsonian volunteers (Jones *et al.*, 1972), indicating that in man, the effect of amantadine may only be demonstrable in the presence of the underlying biochemical abnormalities associated with Parkinson's disease. Secondly, in some behavioural tests, amantadine can be shown to exert a powerful and apparently specific dopaminergic action (Davies *et al.*, 1973; Davies & Redfern, 1974). It is clear that the experimental models used here utilize different facets of normal dopaminergic transmission, and that in order to establish the mode of action of amantadine an experimental model incorporating the essential biochemical lesions of Parkinson's disease may be required.

This work was supported by a CASE studentship; the joint sponsorship of the SRC and Geigy Pharmaceuticals Ltd. is gratefully acknowledged. F.B. is a Parkinson's Disease Society Post Doctoral Research Fellow. We wish to thank the Drug Companies for generous donations of the drugs mentioned in the Methods Section.

## References

ABUZZAHAB, F.S. (1971). Effects of haloperidol on rat brain catecholamines and behaviour. *Fedn Proc.*, 381.

ANDEN, N.E., BUTCHER, S.G., CORRODI, H., FUXE, K & UNGERSTEDT, V. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmac.*, **11**, 303–314.

ANDEN, N.E., FUXE, K. & HOKFELT, T. (1966). The importance of the nervous impulse flow for the depletion of the monoamines from central neurones by some drugs. *J. Pharm. Pharmac.*, **18**, 630–632.

BAK, I.J., HASSLER, R., KIM, J.S. & KATOAKA, K. (1972). Amantadine actions on acetylcholine and GABA in striatum and substantia nigra of rat in relation to behavioural changes. *J. Neurol. Trans.*, **33**, 45–61.

BALDESSARINI, R.J., LIPINSKI, B.F. & CHACE, K.V. (1972). Effects of amantadine HCl on catecholamine metabolism in the brain of the rat. *Biochem. Pharmac.*, **21**, 77–87.

BROWN, F. & REDFERN, P.H. (1974). The effect of amantadine on the turnover of catecholamines in the rat brain. *J. Pharm. Pharmac.*, **26**, 81–82P.

BUUS-LASSEN, J. (1971). Behavioural effect of amantadine in rats after inhibition of monoamine synthesis, storage and receptor interaction. *Acta pharmac. tox.*, **29** Suppl. 4, 30.

BUUS-LASSEN, J. (1973). The effect of amantadine and (+)-amphetamine on motility in rats after inhibition of monoamine synthesis and storage. *Psychopharmacologia*, **29**, 55–64.

COSTALL, B. & NAYLOR, R.J. (1973). The site and mode of action of ET495 for the mediation of stereotyped behaviour in the rat. *Naunyn-Schmeidebergs Arch. Pharmak.*, **278**, 117–133.

DAVIES, J.A. & REDFERN, P.H. (1974). The interaction of dopamine antagonists and amantadine HCl on the acquisition of the conditioned avoidance response in rats. *Neuropharmac.*, **13**, 941–948.

DAVIES, J.A., JACKSON, B. & REDFERN, P.H. (1973). Effect of anti-Parkinsonian drugs on haloperidol-induced inhibition of the conditioned avoidance response in rats. *Neuropharmac.*, **12**, 735–740.

EISENTHAL, R. & CORNISH-BOWDEN, A. (1974). The direct linear plot: a new graphical procedure for estimating enzyme kinetic parameters. *Biochem. J.*, **139**, 715–720.

ERNST, A.M. (1967). Mode of action of apomorphine and dexamphetamine on gnawing compulsion in rats. *Psychopharmacologia*, **10**, 316–323.

FARNEBO, L., FUXE, K., GOLDSTEIN, M., HAMBERGER, B. & UNGERSTEDT, U. (1971). Dopamine and noradrenaline releasing action of amantadine in the central and peripheral nervous system: a possible mode of action in Parkinson's disease. *Eur. J. Pharmac.*, **16**, 27–38.

FLETCHER, E.A. & REDFERN, P.H. (1970). The effect of amantadine on the uptake of dopamine and noradrenaline by rat brain homogenates. *J. Pharm. Pharmac.*, **22**, 957–959.

GLOWINSKI, J. (1972). Some new facts about synthesis, storage and release processes of monoamines in the central nervous system. In *Perspectives in Neuropharmacology*, ed. Snyder, S.H., pp. 349–404. London: Oxford University Press.

HEIKKILA, R.E. & COHEN, G. (1972). Evaluation of amantadine as a releasing agent or uptake blocker for $^3$H-dopamine in rat brain slices. *Eur. J. Pharmac.*, **20**, 156–160.

HEIMANS, R.L.H., RAND, M.J. & FENNESSY, M.R. (1972).

Effects of amantadine on uptake and release of dopamine by a particulate fraction of rat basal ganglion. *J. Pharm. Pharmac.*, **24**, 875–879.

HERBLIN, W.F. (1972). Amantadine and catecholamine uptake. *Biochem. Pharmac.*, **21**, 1993–1995.

JANSSEN, P.A.J. (1967). The pharmacology of haloperidol. *Int. J. Neuropsychiat.*, **3** Suppl. 1, 510–518.

JONES, D.G., TURNBULL, M.J., LENMAN, J.A.R. & ROBERTSON, M.A.H. (1972). Effect of amantadine on the urinary excretion of some monoamines and metabolites in normal and Parkinsonian subjects. *J. Neurol. Sci.*, **17**, 245–253.

MAJ, J., SOWINSKA, H. & BARAN, L. (1972). The effect of amantadine on motor activity and catalepsy in rats. *Psychopharmacologia*, **24**, 296–307.

PAPESCHI, R. (1974). Amanatadine may stimulate dopamine and noradrenaline receptors. *Neuropharmac.*, **13**, 77–84.

RAITERI, M., LEVI, G. & FEDERICO, R. (1974). d-amphetamine and the release of $^3$H-norepinephrine from synaptosomes. *Eur. J. Pharmac.*, **28**, 237–240.

SCATTON, B., CHERAMY, A., BESSON, M.J. & GLOWINSKI, J. (1970). Increased synthesis and release of dopamine in the striatum of the rat after amantadine treatment. *Eur. J. Pharmac.*, **13**, 131–133.

SHELLENBERGER, M.K. & GORDON, J.H. (1971). A rapid simplified procedure for simultaneous assay of norepirephrine, dopamine and 5-hydroxytryptamine from discrete brain areas. *Analyt. Biochem.*, **39**, 356–372.

SNYDER, S.H. & COYLE, J.T. (1969). Regional differences in H$^3$-norepirephrine and H$^3$-dopamine uptake into rat brain homogenates. *J. Pharmac. exp. Ther.*, **165**, 78–86.

STRÖMBERG, U. & SVENSSON, T.H. (1971). Further studies on the mode of action on amantadine. *Acta pharmac. tox.*, **30**, 161–171.

STRÖMBERG, U., SVENSSON, T.H. & WALDECK, B. (1970). On the mode of action of amantadine. *J. Pharm. Pharmac.*, **22**, 959–962.

THORNBURG, J.E. & MOORE, K.E. (1972). A comparison of the locomotor stimulant properties of amantadine and l- and d- amphetamine in mice. *Neuropharmac.*, **11**, 675–682.

VERNIER, V.G., HARMON, J.B., STUMP, J.M., LYNES, T.E., MARVEL, J.P. & SMITH, D.H. (1969). The toxicologic and pharmacologic properties of amantadine hydrochloride. *Toxicol. Appl. Pharmac.*, **15**, 642–665.

VON VOIGTLANDER, P.F. & MOORE, K.E. (1971). Dopamine release from the brain in vivo by amantadine. *Science*, **174**, 408–410.

*(Received March 29, 1976.
Revised June 18, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 569–572

# ON THE FUNCTIONAL COUPLING OF NEUROTRANSMITTER UPTAKE AND RELEASE IN BRAIN

C.W. COTMAN & J.W. HAYCOCK
Department of Psychobiology, University of California, Irvine, California 92717, U.S.A.
W.B. LEVY
Department of Psychology, University of California, Riverside, California 92502, U.S.A.

1 Isolated synaptosomal fractions from mouse forebrains were incubated with [$^{14}$C]-$\gamma$-aminobutyric acid ([$^{14}$C]-GABA). Release of the accumulated label in high potassium solution was measured.

2 The fractional release dependent upon calcium was decreased by raising the concentration of [$^{14}$C]-GABA during labelling but was not affected by altering the time allowed for labelling or the time between labelling and stimulation.

3 These data suggest that extracellular GABA gains rapid access to available intraterminal pools. The relative distribution of the accumulated GABA in different pools can be influenced by the concentration of GABA in the incubation medium but, once 'stored', there is no net redistribution of accumulated GABA in the absence of stimulation.

## Introduction

Synaptosomes exhibit both high-affinity transport systems for the accumulation of neurotransmitters (Kuhar, 1973; Bennett, Mulder & Snyder, 1974) and Ca-dependent secretion systems for the release of endogenously stored transmitter substances (de Belleroche & Bradford, 1972a,b; Blaustein, Johnson & Needleman, 1972; Cotman, Haycock & White, 1976). It has been proposed that the transport systems serve primarily in the termination of synaptic actions of released transmitters (Iversen, 1971). However, it is also possible that uptake systems can procure transmitter for intraterminal stores that specifically supply readily releasable pools (Hughes, 1973; Hughes & Roth, 1974; Ryan & Roskoski, 1975).

The functional coupling, in synaptosomes, between uptake and calcium-dependent secretion of $\gamma$-aminobutyric acid (GABA) and noradrenaline (NA) has been described previously (Levy, Redburn & Cotman, 1973; Levy, Haycock & Cotman, 1974; Cotman, *et al.*, 1976). The secretion processes exhibit properties characteristic of well-studied stimulus-secretion coupling systems, and the accumulation processes that provide transmitter for secretion are Na-dependent. The purpose of the present study was to examine the relationship of transmitter concentration during accumulation and time of accumulation upon the accessibility of newly accumulated transmitter to the release process.

## Methods

After homogenization in 0.32 M sucrose, synaptosomal fractions were isolated from forebrains of male Swiss-Webster mice or corpus striata of Sprague-Dawley rats as described previously (Cotman, *et al.*, 1976). Tissue was resuspended in a HEPES-Ringer solution of the following composition (mM): NaCl 140, HEPES 20, glucose 10, $K_2HPO_4$ 1.5, $MgSO_4$ 1.5, and amino-oxyacetic acid 0.1, pH 7.4 with Tris. All subsequent incubation and release manipulations were performed at room temperature (22–24°C). Tissue aliquots (1 ml, 0.6 mg protein) were plated onto filter units mounted on a vacuum box. Filtrates were collected in vials mounted in a sliding plate within the vacuum box (Levy *et al.*, 1974).

Synaptosomal tissue was labelled with 4-amino-*n*-[U-$^{14}$C] butyric acid (232 mCi/mmol, Amersham/Searle; added to the incubation solution) by perfusing solution through the filter units. After incubation at [$^{14}$C]-GABA concentrations and labelling durations indicated in the Results section, 2 ml washes were administered to the filter units every 30 s (gravity flowthrough for 20 s; negative pressure, approx. 10 mmHg, for 10 seconds). The wash solution consisted of incubation solution with 50 mM KCl added. After washout with 3 × 2 ml washes, matched filter units received either 2 ml of wash solution or 2 ml of wash solution containing 1.5 mM $CaCl_2$.

Radioactivity in the filters and fourth wash filtrates was analyzed by liquid scintillation spectrometry. Ca-dependent release was calculated as the difference between filtrates (in pmol [$^{14}$C]-GABA or % efflux) from filter units receiving calcium and filter units (previously treated identically) receiving wash solution with no calcium added during the fourth wash. In some experiments, [$^3$H]-(–)-NA or [$^3$H]-dopamine (New England) were used.

## Results

As presented in Table 1, increases in either GABA concentration in the incubation solution or the duration of the labelling period increased the amount of [$^{14}$C]-GABA accumulated and the pmol of Ca-dependent [$^{14}$C]-GABA released. When calculated in terms of the fraction of accumulated GABA, however, Ca-dependent [$^{14}$C]-GABA release was affected only by differences in the incubation concentration of GABA. That is, increases in uptake as a function of incubation duration were paralleled by increases in the amount of [$^{14}$C]-GABA release due to calcium. On the other hand, the increases in uptake as a function of GABA concentration were only partially accommodated by increases in the amount released. In separate sets of experiments, Ca-dependent release of [$^3$H]-NA from forebrain synaptosomes and [$^3$H]-dopamine from striatal synaptosomes (0.1 mM pargyline included in incubation solutions) also demonstrated a similar dependence upon transmitter concentration during incubation (Table 1).

Kinetic analyses of [$^{14}$C]-GABA and [$^3$H]-NA accumulation into forebrain synaptosomal fractions and [$^3$H]-dopamine into striatal synaptosomal fractions revealed that even at the highest concentrations investigated in the release studies (10.8, 3.2, 5.0 μM, respectively) the transmitters were accumulated primarily by single, high-affinity sites (GABA: $K_m = 1.4$ μM, $V_{max} = 860$ pmol min$^{-1}$ mg$^{-1}$ protein; NA: $K_m = 2.1$ μM, $V_{max} = 22$ pmol min$^{-1}$ mg$^{-1}$ protein; dopamine: $K_m = 0.3$ μM, $V_{max} =$ 68 pmol min$^{-1}$ mg$^{-1}$ protein) under conditions similar to above.

Within the graphical accuracy of Lineweaver-Burk and Eadie-Hofstee analyses, over 80% of the GABA was accumulated via high-affinity sites at the high concentration in Table 1. [$^3$H]-NA and [$^3$H]-dopamine accumulation described kinetically only a single transport site at the concentrations tested. Although not absolutely sensitive in detecting small kinetic differences between transport sites, these graphical measures should have been able to detect uptake via other sites (low affinity) amounting to, e.g., 60% of the total uptake in the case of [$^{14}$C]-GABA. This would be the extent of low affinity uptake necessary at the higher GABA incubation concentration in order for low affinity (presumably non-readily releasable) accumulation to account for the observed reduction in fractional Ca-dependent release.

**Table 1** Calcium-dependent release as a function of incubation condition

| *Incubation condition* | | | | | |
|---|---|---|---|---|---|
| *Concentration (μM)* | *Duration (s)* | *Post-labelling period (s)* | *Accumulation* (pmol)* | *Baseline efflux (%)* | *Ca-dependent release (%)* |
| [$^{14}$C]-GABA | | | | | |
| 0.43 | 20 | 600 | 29±3 | 1.63±0.11 | 3.56±0.27 |
| 0.43 | 20 | 0 | 33±4 | 3.34±0.35 | 3.30±0.31 |
| 0.43 | 120 | 0 | 228±18 | 2.10±0.18 | 3.12±0.24 |
| 0.43 | 600 | 0 | 906±26 | 1.89±0.06 | 3.23±0.18 |
| 10.8 | 600 | 0 | 4350±1010 | 2.26±0.34 | 1.31±0.25† |
| [$^3$H]-NA | | | | | |
| 0.06 | 600 | 0 | 3.8±0.1 | 3.50±0.40 | 6.09±0.64 |
| 3.2 | 600 | 0 | 79±2 | 8.34±0.25 | 2.05±0.14† |
| [$^3$H]-dopamine | | | | | |
| 0.05 | 600 | 0 | 49±4 | 1.71±0.08 | 9.36±0.40 |
| 5.0 | 600 | 0 | 297±19 | 11.65±0.42 | 4.25±0.31† |

* Totals on filter immediately prior to stimulation; calculated on the basis of initial specific activities of labelled transmitter; for [$^{14}$C]-GABA and [$^3$H]-NA, pmol/0.6 mg forebrain synaptosomal protein and for [$^3$H]-dopamine, pmol/0.3 mg striatal synaptosmal protein.

† $P < 0.05$, two-tailed $t$-test for inherently paired samples.

All values represent the means and s.e. mean for four determinations.

## Discussion

For widely varying amounts of transmitter uptake, fractional release should be diagnostic for variations in the relative distribution of the accumulated transmitter with respect to readily releasable pools (defined here by the 20 s stimulation period). Our findings indicate that incubations as short as 20 s label readily releasable pools in equivalent proportion to incubations up to 30 times longer. Thus the accumulated transmitter not only has access to the readily releasable pools but this access is rapid. Although availability to the release process is rapid, the GABA accumulated during a 20 s incubation did not demonstrate a detectable redistribution with respect to the readily releasable pools over a 10 min period.

Following a 10 min incubation with 0.43 µM [$^{14}$C]-GABA, % Ca-dependent release of both labelled and endogenous GABA are similar (Cotman, *et al.*, 1976), and the specific activities (sp. act.) of the tissue and released GABA are similar. In that brain GABA levels are relatively insensitive to extra-terminal GABA and amino-oxyacetic acid concentrations *in vitro* (Starr & Tanner, 1975; unpublished observations), alterations of % release of [$^{14}$C]-GABA for the conditions investigated above should reflect alterations of the released:tissue ratio of GABA sp. act. The decrease in % [$^{14}$C]-GABA release after incubation at the high GABA concentration suggests: (1) that the sp. act. of the released GABA did not increase in proportion to the increase in tissue GABA sp. act. and (2) that a greater percentage of the labelled GABA (presumably accumulated by the same set of membrane transport sites) was being stored in less readily releasable stores. As such, these data implicate multiple *intra*terminal GABA pools that are kinetically distinct both in terms of (1) affinities and/or storage capacities and in terms of (2) participation in stimulus-secretion coupling processes.

These data are similar to those reported by Hughes (1973) for [$^{3}$H]-NA release from rabbit vasa deferentia in that higher incubation concentrations produced a greater amount, but lower fraction, of [$^{3}$H]-NA release. However, in apparent contrast to the present report, the accumulated NA in the vas was released in preference to endogenous NA after incubation at lower NA concentrations. The dynamics of NA in the vas and GABA in the brain may be quite disparate but comparison between these systems is difficult considering the differences in tissue (whole organ *vs* isolated fraction) and temporal aspects (h *vs* min) of the experiments.

Ryan & Roskoski (1975) recently reported that newly accumulated GABA was released in preference to endogenous GABA from isolated synaptosomes. However, the present data showing a constant fractional release over both duration of incubation and incubation-stimulation interval and previous data showing equivalence of tissue and released GABA sp. act. (Cotman, *et al.*, 1976) indicate no such preferential release of accumulated GABA for the conditions described above (notably, without prior stimulation). Ryan & Roskoski (1975) stimulated release by simultaneously elevating K and Ca. We find that elevated K pulses alone (in the absence of Ca) can induce a preferential efflux of labelled GABA from synaptosomes and that without subtraction of this baseline efflux (notably high for K pulses) spurious inferences regarding Ca-dependent release can be made (Cotman, *et al.*, 1976). We have been able to obtain preferential release of accumulated GABA only under conditions of prior stimulation (Levy, Haycock & Cotman, 1976).

These data suggest a physiological role for reuptake processes in maintaining synaptic transmission in brain. The rapidity with which accumulated GABA has access to readily releasable pools makes possible a direct coupling between reuptake and stimulus-secretion coupling processes. The absolute contribution of this reaccumulated transmitter to secretion may be controlled by the extraterminal concentration of transmitter immediately subsequent to release. One hypothesis is that, at higher concentrations, relatively more transmitter is accumulated into those compartments within the terminal where it has a less immediate access to secretory processes (e.g., extravesicular or less readily releasable vesicular stores).

This work was supported by Research Grants NS 08597 to C.W.C. and BMS 75-18089 to W.B.L. and an NSF Predoctoral Fellowship to J.W.H. The data were taken from portions of a doctoral thesis by J.W.H.

## References

DE BELLEROCHE, J.S. & BRADFORD, H.F. (1972a). Metabolism of beds of mammalian cortical synaptosomes: Response to depolarizing influences. *J. Neurochem.*, **19**, 585–602.

DE BELLEROCHE, J.S. & BRADFORD, H.F. (1972b). The stimulus-induced release of acetylcholine from synaptosome beds and its calcium dependence. *J. Neurochem.*, **19**, 1817–1819.

BENNETT, J.P., MULDER, A.H. & SNYDER, S.H. (1974). Neurochemical correlates of synaptically active amino acids. *Life Sci.*, **15**, 1045–1056.

BLAUSTEIN, M.P., JOHNSON, E.M., Jr. & NEEDLEMAN, P. (1972). Calcium-dependent norepinephrine release from presynaptic nerve endings *in vitro*. *Proc. Nat. Acad. Sci. USA*, **69**, 2237–2240.

COTMAN, C.W., HAYCOCK, J.W. & WHITE, W.F. (1976). Stimulus-secretion coupling processes in brain: Analysis of noradrenaline and gamma-aminobutyric acid release. *J. Physiol., Lond.*, **254**, 475–505.

HUGHES, J. (1973). Differential labelling of intraneuronal stores with different concentrations of (–)-$^3$H-noradrenaline. *Br. J. Pharmac.*, **47**, 428–430.

HUGHES, J. & ROTH, R.H. (1974). Variation in noradrenaline output with changes in stimulus frequency and train length: Role of different noradrenaline pools. *Br. J. Pharmac.*, **51**, 373–381.

IVERSEN, L.L. (1971). Role of transmitter uptake mechanisms in synaptic neurotransmission. *Br. J. Pharmac.*, **41**, 571–591.

KUHAR, M.J. (1973). Neurotransmitter uptake: A tool in identifying neurotransmitter-specific pathways. *Life Sci.*, **13**, 1623–1634.

LEVY, W.B., HAYCOCK, J.W. & COTMAN, C.W. (1974). Effects of polyvalent cations on stimulus-secretion coupling of $^{14}$C-GABA from isolated brain synaptosomes. *Molec. Pharmac.*, **10**, 438–449.

LEVY, W.B., HAYCOCK, J.W. & COTMAN, C.W. (1976). Stimulation-dependent depression of readily releasable neurotransmitter pools in brain. *Brain Res.*, (in press).

LEVY, W.B., REDBURN, D.A. & COTMAN, C.W. (1973). Stimulus-coupled secretion of $\gamma$-aminobutyric acid from rat brain synaptosomes. *Science*, **181**, 676–678.

RYAN, L.D. & ROSKOSKI, R. (1975). Selective release of newly synthesized and newly captured GABA from synaptosomes by potassium depolarization. *Nature, Lond.*, **258**, 254–256.

STARR, M.S. & TANNER, T. (1975). Effect of aminooxyacetic acid, ethanolamine-*o*-sulphate and GABA on the contents of GABA and various amines in brain slices. *J. Neurochem.*, **25**, 573–577.

*(Received March 30, 1976.*
*Revised June 14, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 573–581

# THE LOCAL ANTINOCICEPTIVE AND TOPICAL ANTI-INFLAMMATORY EFFECTS OF PROPYL GALLATE IN RODENTS

WENDY J. McDONALD-GIBSON, S.A. SAEED & C. SCHNEIDER
Research Division, Miles Laboratories Ltd, Stoke Court, Stoke Poges, Slough, SL2 4LY

1 In common with several anti-inflammatory, analgesic, local anaesthetic and antioxidant drugs, propyl gallate *in vitro* inhibited the biosynthesis of prostaglandin $E_2$ and $F_{2\alpha}$ from arachidonic acid by a prostaglandin synthetase from bull seminal vesicles.

2 In common with analgesic drugs, propyl gallate reduced the ability of arachidonic acid, acetylcholine or acetic acid to cause abdominal constrictions in mice.

3 Using a new method of evaluating anti-inflammatory activity, we demonstrated the effectiveness of aspirin or indomethacin given subcutaneously before u.v. irradiation of guinea-pig ears, the prophylactic action of topically applied sunscreen agents and the therapeutic value of bufexamac and propyl gallate applied after irradiation.

## Introduction

Propyl gallate is used as an antioxidant to stabilize oils, fats and cosmetics against oxidative deterioration. Previous studies (Tappel, Lundberg & Boyer, 1953 and Saeed, S.A., unpublished) have shown that propyl gallate inhibits hydroperoxidation of several polyunsaturated fatty acids. It has also been reported (Nugteren, Beerthuis & Van Dorp, 1966) that propyl gallate inhibits the conversion of di-homo-$\gamma$-linolenic acid to prostaglandin $E_1$ by microsomal particles of sheep vesicular glands. Otomo & Fujihira (1970) suggested that several non-steroidal anti-inflammatory drugs (NSAID) stabilize erythrocyte membranes against hydrogen peroxide-induced lipid peroxidation by 'mopping up' free radicals or peroxides. Subsequently, Vane and co-workers (Ferreira, Moncada & Vane, 1971; Smith & Willis, 1971; Vane, 1971; Flower, Gryglewski, Herbaczynska-Cedro & Vane, 1972) showed that clinically attainable plasma concentrations of NSAID inhibit prostaglandin biosynthesis. Since lipid peroxides and prostaglandins are important in the inflammatory process, we compared inhibition of prostaglandin biosynthesis by propyl gallate with several anti-inflammatory drugs. These experiments with bull seminal vesicle prostaglandin synthetase showed that propyl gallate was seven times as potent as aspirin. We also investigated the antinociceptive and topical anti-inflammatory activity of propyl gallate in laboratory rodents.

## Methods

### *Inhibition of prostaglandin synthetase*

The effects of propyl gallate and several other classes of drugs on prostaglandin biosynthesis *in vitro* were measured against prostaglandin synthetase of bull seminal vesicles (BSV) prepared as described previously (Collier, McDonald-Gibson & Saeed, 1974). Experiments were performed by incubation of an appropriate amount of the test substance in a standard assay mixture (2.0 ml) containing 50 mM phosphate buffer pH 7.4, 0.66 mM reduced glutathione, 0.09 mM hydroquinone, 5.25 mM disodium edetate (EDTA) and 500 µl of the BSV prostaglandin synthetase preparation. The reaction was started by the addition of sodium arachidonate (final concentration 0.061 mM) and the tubes aerated at 37°C with gentle shaking. After 12 min, the reaction was terminated by adding 2.0 ml of 0.2 M citric acid and the mixture was extracted with 16 ml ethyl acetate. After evaporation of ethyl acetate, the residue was re-dissolved in Krebs solution and assayed on rat stomach strip in the presence of antagonists of acetylcholine, catecholamines, histamine and 5-hydroxytryptamine. All experiments were carried out in duplicate with appropriate controls.

### *Antinociceptive tests*

Generally, methods were as described by Collier, Dinneen, Johnson & Schneider (1968) and Collier, Saeed, Schneider & Warren (1973). Arachidonic acid (100 µg/ml) was incubated with propyl gallate (0–2 mg/ml). The incubate was tested intraperitoneally for its ability to induce abdominal constrictions in mice. Also, mice were pretreated with propyl gallate 1 to 4 mg/kg intraperitoneally 5 min before challenge with arachidonic acid (1 mg/kg) close to the site of the first injection, propyl gallate being injected into the midline of the abdomen, arachidonic acid given slightly lower and to the right of this site. In a second batch of experiments, the mice were treated with propyl gallate (40 mg/kg intraperitoneally or subcutaneously) 5 min before intraperitoneal challenge with acetylcholine bromide (3.2 mg base/kg). Third, propyl gallate (10 or 40 mg/kg) intraperitoneally was given 5 min before a solution of acetic acid (75 mg/kg, i.p.) or acetylcholine bromide respectively. Fourth, sodium aspirin (40 mg as aspirin)/kg was given subcutaneously 10 min before challenge and propyl gallate (40 mg/kg) given intraperitoneally 2 min before challenge by acetylcholine or acetic acid.

In these abdominal constriction tests, solutions for injection were coded so that the observer of the abdominal constriction responses was unaware of the drug treatment given. The appropriate vehicles were always given as controls. The statistical significance of results was found using 2 × 2 Contingency Tables (Finney, Latscha, Bennett & Hsu, 1963).

### *Topical anti-inflammatory test*

A preliminary account of the method was given by McDonald-Gibson & Schneider (1974). Individually marked Hartley strain male albino guinea-pigs weighing 200–300 g were used. The dorsal surfaces of both ears were depilated by Nair cream, and the cream removed by washing in tap water. Twenty hours later, a group of 4 guinea-pigs was placed in a cage 40 cm long × 15 cm wide × 10 cm high, separated from each other by wire mesh barriers. Test substances were applied to one ear using 100–150 mg of water miscible cream (Acid Mantle cream or occasionally Carbowax cream). The other ear received vehicle alone. In each experiment involving four guinea-pigs, two right ears and two left ears were treated with the test substance. Twenty min after each application, all four guinea-pigs were placed for 30 min under a 20 W u.v. strip-light (57 cm long; 8 cm above their heads). The lamp was switched on for 30 min before irradiating the animals. In unprotected animals, irradiation caused a marked increase in ear temperature, erythema, delayed oedema and blister formation. Food and water were withheld until observations on ear temperatures were complete (usually 4 h after irradiation). In other experiments test substances were applied immediately following irradiation or injected subcutaneously 20 min before irradiation. In one experiment sodium aspirin was injected 2 h and 6 h after irradiation.

Estimates of erythema were made subjectively by two independent observers, each usually comparing the test and control ear. When drugs were given subcutaneously, comparisons were made between test and control guinea-pigs. From 5–280 min after irradiation, ear temperature was monitored with a thermistor probe set in plasticine firmly on the dorsal surface of each ear in turn for 2.5 minutes. Temperature was measured via a Wheatstone bridge and a twin-channel Devices polygraph with a DC2-D pre-amplifier.

Ear thickness was measured by a micrometer screw gauge immediately before application of test substance and 24 h after irradiation. Oedema was taken as being proportional to the increase in ear thickness. Blister formation was assessed subjectively 5–7 days after irradiation by two independent observers.

In experiments in which ear temperature was measured, the animals were kept at a laboratory temperature of 24–26°C; in others they were kept at 20–23°C. In most experiments, the guinea-pigs were returned to their home cages immediately after irradiation and removed for measurement of ear temperature or ear-lobe thickness.

Statistical analyses of the results of the experiments in guinea-pigs were carried out by Mr L.C. Dinneen in this department using conventional methods.

### *Materials*

Drugs were obtained from various pharmaceutical manufacturers (as indicated) and were prepared in aqueous solution either as the free acid or base or as the sodium or hydrochloride salt respectively. All drugs were of analytical grade; acetic acid (BDH), acetylcholine bromide (Sigma), Acid Mantle cream (Dome), amidopyrine (BDH), arachidonic acid (99% pure, Sigma), benzocaine (Sigma), benzydamine (Angelini Franchesco), bufexamac (butoxyphenyl-acetyl-hydroxamic acid; Lederle), butylated hydroxyanisole (BHA; Sigma), Carbowax cream (Dome), citric acid (BDH), ethyl acetate (BDH), ethylenediamine tetra-acetate sodium (EDTA; Hopkin & Williams), flufenamic acid (Parke-Davis), glutathione (reduced form; Sigma), hyoscine hydrobromide (BDH), hydroquinone (Koch-Light), ibuprofen (Boots), indomethacin (Merck, Sharp & Dohme), meclofenamic acid (Parke-Davis), mefenamic acid (Parke-Davis), mepyramine maleate (May & Baker), methysergide bimaleate (Sandoz), Nair cream (Carter Wallace), nordihydroguaiaretic acid (NDGA; Fluka, A.G.), oxyphenbutazone (Geigy), para amino benzoic acid (PABA; Sigma), paracetamol (Koch-Light), phenazone (L. Light & Co.), phenoxybenzamine (Menley & James), phenylbutazone (Geigy), procaine hydrochloride (Sigma), propranalol hydrochloride (ICI), propyl gallate (Sigma), prostaglandin $E_2$ (Ono), prostaglandin $F_{2\alpha}$ (Ono), sodium acetylsalicylate (Miles), sodium chloride (BDH), sodium phosphate (BDH), Uval (2-hydroxy-4-methoxy-benzophenone-5-sulphonic acid; Dome), Vitamin E (Sigma).

## Results

### *Inhibition of prostaglandin synthetase*

As incubation of arachidonic acid with bull seminal vesicle homogenate (plus co-factors glutathione and hydroquinone) produced an average of 44 times more prostaglandin $E_2$ than $F_{2\alpha}$ results are expressed in terms of prostaglandin $E_2$ equivalents. Rank order of potency inhibiting prostaglandin biosynthesis *in vitro* correlated well with known analgesic activity in man and antinociceptive activity in mice (for references see Collier *et al.*, 1968). Results are shown in Table 1. Each of the 19 drugs incubated with arachidonic acid, bovine seminal vesicle prostaglandin synthetase preparation plus co-factors glutathione and hydroquinone reduced prostaglandin biosynthesis in a dose-related manner. Meclofenamate was the most potent drug tested, and although propyl gallate was 1000 times less potent, it was approximately 7 times more potent than aspirin and only slightly less potent than oxyphenbutazone in this system. Some substances other than the well known anti-inflammatory agents inhibited prostaglandin biosynthesis. These included the antioxidants butylated hydroxyanisole and nordihydroguaiaretic acid and the sunscreen agent 2-hydroxy-4-methoxy-benzo-phenone-5-sulphonic acid.

### *Antinociceptive activity*

Table 2 shows that the ability of arachidonic acid (100 µg/ml) to induce abdominal constrictions in mice was significantly ($P < 0.01$) less after it had been incubated with 2 mg/ml of propyl gallate. Also, pretreatment of mice with 4 mg/kg propyl gallate intraperitoneally, significantly ($P < 0.005$) inhibited abdominal constrictions to a subsequent intraperitoneal challenge with arachidonic acid (Table 3). Furthermore, treatment of mice with propyl gallate, 40 mg/kg intraperitoneally 5 min before challenge, reduced abdominal constrictions in response to acetylcholine ($P < 0.01$). Propyl gallate, 10 mg/kg intraperitoneally 5 min before challenge, reduced abdominal constrictions induced by intraperitoneal acetic acid. In addition, propyl gallate 10 or 40 mg/kg significantly reduced diarrhoea ($P < 0.05$) after arachidonic acid or

**Table 1** Inhibition of prostaglandin biosynthesis *in vitro*

| *Drug* | *$IC_{50}$ (µM)* |
|---|---|
| *Non-steroidal anti-inflammatory* | |
| Meclofenamate | 0.11 |
| Flufenamate | 0.5 |
| Indomethacin | 1.2 |
| Mefenamate | 2.2 |
| Ibuprofen | 6.8 |
| Phenylbutazone | 13.0 |
| Oxyphenbutazone | 41.0 |
| Benzydamine | 410 |
| Amidopyrine | 518 |
| Phenazone | 637 |
| Aspirin | 692 |
| Paracetamol | 2516 |
| *Local anaesthetic and sunscreens* | |
| Uval (2-hydroxy-4-methoxy-benzophenone-5-sulphonic acid) | 227 |
| Benzocaine | 569 |
| Para amino benzoic acid (PABA) | 2828 |
| *Antioxidants* | |
| Nordihydroguaiaretic acid | 42 |
| Butylated hydroxyanisole | 52 |
| Propyl gallate | 103 |
| Vitamin E | 5804 |

$IC_{50}$; concentration which inhibits prostaglandin biosynthesis by 50%. The test drugs in aqueous solution were incubated with sodium arachidonate, glutathione, hydroquinone, EDTA and bull seminal vesicle homogenate in sodium phosphate buffer (see text for details). After extraction and re-constitution in Krebs solution the prostaglandins were assayed against prostaglandin $E_2$ on rat stomach strip using methods described by Collier *et al.* (1974).

**Table 2** Inhibition by propyl gallate of the abdominal constriction response induced by arachidonic acid in the mouse

| *Concentration of propyl gallate in incubate (mg/ml)* | *No. of mice* | *No. of mice responding within min* | | | *No. of responses by min* | | |
|---|---|---|---|---|---|---|---|
| | | *0–0.5* | *0.5–2* | *2–10* | *0–0.5* | *0.5–2* | *2–10* |
| None | 50 | 12 | 16 | 20 | 20 | 37 | 84 |
| 0.25 | 35 | 2* | 5 | 9** | 2 | 7 | 17 |
| 0.40 | 5 | 1 | 2 | 5 | 1 | 8 | 38 |
| 0.50 | 10 | 0 | 0* | 0** | 0 | 0 | 0 |
| 1.00 | 45 | 0** | 1** | 8 | 0 | 1 | 23 |
| 2.00 | 5 | 0 | 0 | 4 | 0 | 0 | 6 |

Abdominal constrictions were induced by intraperitoneal injection of an incubate containing arachidonic acid (100 μg/ml), mouse peritoneal exudate (0.2 ml), propyl gallate (0–2 mg/ml), in 50 mM phosphate buffer containing ethanol (5% v/v), glutathione (50 μg/ml) and hydroquinone (10 μg/ml).
* $P<0.05$; ** $P<0.01$.

**Table 3** Inhibition by propyl gallate of abdominal constrictions induced by arachidonic acid, acetylcholine or acetic acid in the mouse

| *Intraperitoneal treatment* | *No. of mice* | *% of mice responding within* | | |
|---|---|---|---|---|
| | | *0–0.5 min* | *0.5–2 min* | *2–10 min* |
| AA + control vehicle | 40 | 10 | 25 | 55 |
| AA + propyl gallate (25 μg/ml) | 35 | 6 | 5 | 23** |
| AA + propyl gallate (100 μg/ml) | 35 | 0 | 3* | 6** |
| AA 5 min after control vehicle | 25 | 44 | 48 | N.T. |
| AA 5 min after propyl gallate (1 mg/kg) | 25 | 41 | 20* | N.T. |
| AA 5 min after propyl gallate (4 mg/kg) | 20 | 0*** | 0* | N.T. |
| ACh 5 min after control vehicle | 20 | 5 | 85 | 6/10 |
| ACh 5 min after propyl gallate (40 mg/kg) | 20 | 0 | 10*** | 1/10* |
| Acac 5 min after control vehicle | 40 | 0 | 3 | 48 |
| Acac 5 min after propyl gallate (10 mg/kg) | 30 | 0 | 0 | 10** |

Methods were those described by Collier *et al.* (1968).
AA = arachidonic acid, 1 mg 10 $ml^{-1}$ $kg^{-1}$; ACh = acetylcholine bromide, 3.2 mg base 10 $ml^{-1}$ $kg^{-1}$; Acac = acetic acid, 75 mg 10 $ml^{-1}$ $kg^{-1}$. Propyl gallate was dissolved in sodium phosphate buffer, 0.05 M, pH 7.0. NT = not tested.
* $P<0.05$; ** $P<0.01$: *** $P<0.005$.

acetylcholine challenge respectively, and also appeared to protect some mice from the immobilizing effect of arachidonic acid. Results in Table 4 show that sodium aspirin and propyl gallate together reduced abdominal constrictions to acetylcholine more effectively than did either drug alone.

In contrast, propyl gallate 10 or 40 mg/kg orally or subcutaneously in 4 groups of 10 mice had no antinociceptive activity against arachidonic acid.

### *Topical anti-inflammatory activity*

Using the method of Tonelli, Thibault & Ringler (1965), B.M. Phillips (Miles Laboratories Inc., Elkhart, Indiana, USA, personal communication) found that propyl gallate showed significant topical anti-inflammatory activity, having a potency 0.03 relative to hydrocortisone.

Tables 5, 6 and 7 show results of tests of anti-inflammatory activity. Topically, propyl gallate at 10% w/w before and immediately after irradiation of guinea-pig ears significantly reduced ($P < 0.05$) the heat and erythema observed for up to 4 h, the oedema that develops by 24 h and the blister formation that develops within 5 days. Lower concentrations of propyl gallate had progressively less effect, but even 1.25% w/w significantly ($P < 0.01$) reduced ear heat. Propyl gallate 5 or 10% w/w was also significantly effective ($P < 0.01$) when applied only after irradiation; at 10% w/w it reduced ear heat, erythema, oedema and blister formation.

Other substances which reduced inflammatory responses when applied before and after u.v. irradiation were the sunscreen Uval, the antioxidants butylated hydroxyanisole (BHA) and nordihydroguaiaretic acid (NDGA), each being less effective than propyl gallate. Mixtures of propyl gallate with NDGA or with PABA were also effective. Soluble buffered aspirin given subcutaneously also reduced ear heat, erythema and oedema.

## Discussion

Although there is a good rank order correlation between inhibition of prostaglandin synthetase and analgesic potency of non-steroidal anti-inflammatory drugs, absolute potencies against prostaglandin synthetase from bovine seminal vesicles do not correlate well with absolute potency of NSAID in the treatment of inflammatory conditions such as rheumatoid arthritis in man. Therapeutic doses of aspirin for example are not 567 × those of indomethacin. Several antioxidants also inhibited, in a dose-related manner, prostaglandin biosynthesis.

The expected antinociceptive activity of propyl gallate was confirmed in mice. High concentrations of propyl gallate when incubated *in vitro* with arachidonic acid lessened the ability of arachidonic acid to induce abdominal constrictions. Simultaneous or prior administration of propyl gallate also inhibited abdominal constrictions induced by arachidonic acid. Responses to acetylcholine or to acetic acid were inhibited by high doses of propyl gallate. When administered with sodium aspirin there was an apparent additive antinociceptive effect, which would be expected if the drugs had similar modes of action.

The antinociceptive actions of propyl gallate in the mouse might be due at least in part to a local anaesthetic action. Hendershot & Forsaith (1959) thought that procaine given intraperitoneally exerted a local anaesthetic effect when blocking abdominal con-

**Table 4** Inhibition by propyl gallate plus sodium aspirin of abdominal constrictions induced by acetylcholine or by acetic acid in the mouse

| *Subcutaneous treatment 10 min before challenge* | *Intraperitoneal treatment 2 min before challenge* | *Intraperitoneal challenge* | *No. of mice* | *No. of mice responding within* | | |
|---|---|---|---|---|---|---|
| | | | | *0–30 s* | *0.5–2 min* | *2–10 min* |
| NAS | Propyl gallate | ACh | 15 | 0 | 0*** | NT |
| Control | Control | ACh | 15 | 4 | 12 | NT |
| NAS | Propyl gallate | ACh | 15 | 0 | 0*** | NT |
| NAS | Control | ACh | 10 | 0 | 9 | NT |
| NAS | Propyl gallate | ACh | 10 | 0 | 0*** | NT |
| Control | Propyl gallate | ACh | 10 | 1 | 7 | NT |
| NAS | Propyl gallate | Acac | 5 | 0 | 0 | 4 |
| NAS | Control | Acac | 15 | 0 | 0* | 2 |
| NAS | Propyl gallate | Acac | 5 | 0 | 0 | 4 |
| Control | Propyl gallate | Acac | 5 | 0 | 0 | 5 |

NAS; sodium aspirin 40 mg (as acetylsalicylic acid)/kg; Propyl gallate 40 mg/kg. Other details as in Table 3.

**Table 5** Inhibition by drugs of heat, erythema and oedema of guinea-pig ears irradiated by ultra-violet light

| *Treatment* | *Concentration of propyl gallate or other test drug* | *Note* | *Mean increase in temperature (°C) of control ears compared with treated ears at times after exposure to u.v. light* | | | | *Proportion of treated ears with less erythema than controls* | *Proportion of treated ears with less oedema than controls* |
|---|---|---|---|---|---|---|---|---|
| | | | *20–40 min* | *41–60 min* | *100–120 min* | *200–240 min* | | |
| Propyl gallate | 10% | | 4.59*** | 5.33*** | 5.94*** | 4.01*** | NT | 4/4 |
| Propyl gallate | 10% | a | 3.72** | 3.25* | 5.10* | 4.80*** | 4/4 | 5/5 |
| Propyl gallate | 10% | b | 2.30** | 2.40** | 1.28** | 2.25** | 4/4 | 4/4 |
| Propyl gallate | 10% | c | 3.03*** | 3.48*** | 3.32*** | 1.00*** | 12/12 | 11/12 |
| Propyl gallate | 10% | a,c | 1.90 | 2.23 | 3.73 | 1.30 | 4/4 | 4/4 |
| Propyl gallate | 5% | a | 3.86*** | 3.96*** | 5.10*** | 2.36* | 5/5 | 5/5 |
| Propyl gallate | 2.5% | a | 3.62** | 3.86** | 3.42** | 1.28*** | 4/4 | 1/1 |
| Propyl gallate | 1.25% | a | 1.54 | 1.68 | 2.80*** | 0.88 | 4/4 | 4/4 |
| Propyl gallate | 0.625% | | 0.08 | 0.38$^{d}$ | 0.38 | 0.28 | 0/4 | 1/4 |
| Propyl gallate | 0.5% | | 0.15$^{d}$ | 1.05 | NT | 0.30 | 0/2 | 0/2 |
| Propyl gallate | 10% | e | 4.85* | 5.80*** | 5.88*** | NT | 4/4 | 4/4 |
| Propyl gallate | 5% | c | 3.40** | 4.40*** | 4.47*** | 0.93* | 3/3 | NT |
| Uval | 10% | | 5.81*** | 6.19*** | 6.70*** | 8.20*** | 4/4 | 4/4 |
| Uval | 10% | c | 0.33*$^{d}$ | 0.14** | 0.07 | 0.07 | 0/7 | 1/7 |
| BHA | 10% | | 0.76*** | 1.08*** | 1.24** | 0.98** | 12/12 | 7/8 |
| NDGA | 10% | | 0.73 | 0.70* | 0.03 | 0.10$^{d}$ | 0/4 | 2/4 |
| Propyl gallate and NDGA | 0.5%<br>9.5% | f | 2.83* | 3.80*** | 3.70 | 1.78* | 4/4 | 4/4 |
| Sodium aspirin | 150 mg/kg | g | 3.90*** | 4.50** | 4.30** | 1.00 | 4/4 | 2/2 |

Uval contains 2-hydroxy-4-methoxy-benzophenone-5-sulphonic acid; BHA is butylated hydroxyanisole; NDGA is nordihydroguaiaretic acid; PABA is para amino benzoic acid. NT indicates not tested. Concentration refers to % w/w applied to test ear.

Notes (a) Propyl gallate was added to warmed vehicle. When the cream had cooled to room temperature it was applied to the guinea-pig ears. (b) Drug was applied 10 min before irradiation. (c) Cream applied only after irradiation. (No pretreatment). (d) Increased temperature of treated ears compared with controls. (e) Carbowax cream was used, instead of Acid Mantle cream. (f) Mixture of two substances. (g) Solution of sodium acetylsalicylate (150 mg aspirin/kg) given subcutaneously 30 min before irradiation.

* $P \leqslant 0.05$; ** $P \leqslant 0.02$; *** $P \leqslant 0.01$.

**Table 6** Inhibition by topically-applied drugs of oedema of guinea-pig ears irradiated by ultra-violet light

| *Treatment* | *Concentration % w/w* | *Note* | *Mean difference in thickness (μm) between treated and control ears 24 h after exposure to u.v. light* | *Statistical significance* | *Proportion of treated ears with less oedema* than controls* |
|---|---|---|---|---|---|
| Propyl gallate | 10 | | +165.1[i] | NS | 3/4 |
| Propyl gallate | 10 | c | −162.1 | $P<0.05$ | 7/8 |
| Propyl gallate | 10 | e | −393.7 | $P<0.002$ | 4/4 |
| Propyl gallate | 0.5 | | +114.3[i] | NS | 0/2 |
| Propyl gallate | 0.625 | | −19.05 | NS | 2/4 |
| Uval | 10 | c | +127.0[i] | $P<0.05$ | 5/7[i] |
| BHA | 10 | | −251.0 | $P<0.05$ | 8/8 |
| NDGA | 10 | | −6.35 | NS | 2/4 |
| Propyl gallate +<br>NDGA | 0.5<br>9.5 | f | −254.0 | NS | 4/4 |
| PABA | 10 | | −355.6 | $P<0.02$ | 4/4 |

* Difference between control and treated ears 24 h after irradiation. The figures in column 5 analysed statistically have used measurements taken both before and after 24 h irradiation. i = more oedema in treated ear. NS = not significant.
Other details as in Table 5.

strictions elicited by phenylbenzoquinone. Modak & Rao (1971) found that in the frog, rabbit and guinea-pig, propyl gallate is as potent a local anaesthetic agent as procaine. Although we found benzocaine to inhibit prostaglandin biosynthesis, procaine had little activity.

Our results in mice suggest that propyl gallate has little clinical potential as a subcutaneous or oral drug in man. However, its topical application to skin may prove useful in preventing inflammation caused by sunburn (Saeed, Schneider & Phillips, 1974) as suggested by the guinea-pig erythema test. The modifications that we made in this test have several advantages. Heat and oedema can be quantified objectively, and blister formation can also be assessed. Each animal may be used as its own control, drugs can be given parenterally or applied topically in various vehicles, and the animals do not need to be restrained manually during irradiation. Furthermore, hyperalgesia could probably also be measured, if necessary.

The reduction of heat, oedema, erythema and blister formation of the ears was not due solely to absorption of u.v. light by propyl gallate, since protection also occurred when the drug was applied after irradiation.

In man, erythema is induced most by u.v. radiation of 296.7 nm (Giese, Christensen & Jeppson, 1950; Knox, Guin & Cockerell, 1957). If this wavelength is as damaging to guinea-pigs as it is to man, the apparent anti-inflammatory activity of Uval, BHA, NDGA and PABA may be due only to absorption of u.v. light; Uval was not effective when applied only after irradiation. Clinically it is applied as a sunscreen before exposure to sunlight.

The anti-inflammatory activity of parenterally administered aspirin or indomethacin and of bufexamac (a new non-steroidal topical anti-inflammatory drug (Grigoriu, 1972)) indicates that the new method is useful for assessing anti-inflammatory activity of aspirin-like drugs.

Snyder & Eaglstein (1974) found that a single application of a 2.5% solution of indomethacin decreased the redness, warmth and tenderness of sunburned human skin (Kesten, 1956) for 24 h or longer, and was more effective than a corticosteroid cream. However, they did not state whether the indomethacin was applied before or after u.v. radiation.

Kahn & Curry (1974) reported that propyl gallate was an extremely effective sunscreen. They found, however, that it was a strong contact sensitizer, in agreement with the findings of B.M. Phillips (personal communication). Recently, Lewis (1976) showed that propyl gallate has topical anti-inflammatory activity in the rat, thus confirming our results in the guinea-pig (McDonald-Gibson & Schneider, 1974).

The use of propyl gallate might be justified in the treatment of painful burns, in view of the report by Jelenko & Wheeler (1972) that various antioxidants, when mixed with ethyl linoleate, prolonged its efficacy and appeared to act as oxygen acceptors. These investigators claimed that the antioxidants had potential use in burn therapy as topical agents to control excessive post-burn evaporative water loss and perhaps hypermetabolism after burns.

Propyl gallate may also potentiate the effect of adrenaline which reduces erythema partly by vasoconstrictor activity. Grunwaldt (1972) showed that alkyl gallates effectively prevented the catabolism of adrenaline and noradrenaline which were used in the treatment of glaucoma. Greengard & Petrack (1972) claimed that alkyl gallates plus adrenaline or noradrenaline in a preparation suitable for inhalation, were useful for treating bronchospasms. Also, Cash, Petrack & Weiner (1971) claimed that propyl gallate increased the anti-Parkinson activity of L-DOPA.

**Table 7** Inhibition by topically applied propyl gallate of blister formation of guinea-pig ears irradiated by ultra-violet light

| *Treatment with propyl gallate* | | *Concentration % w/w* | *Proportion of control ears with greater* blister formation than treated ears 4 or 5 days after exposure to u.v. light* |
|---|---|---|---|
| *Before u.v.* | *After u.v.* | | |
| + | + | 0.625 | 1/4 |
| + | + | 10 | 4/4 |
| + | + | 10$^{e}$ | 4/4 |
| – | + | 10 | 4/4<br>slight protection |

* Determined subjectively by two observers; e = in Carbowax cream; + = propyl gallate applied; – = propyl gallate not applied.
Other details as in Table 5.

Propyl gallate may exert some of its anti-inflammatory action by inhibiting the effects of bradykinin as demonstrated on the guinea-pig ileum by Posati, Fox & Pollansch (1970).

We thank Mr L.C. Dinneen for statistical help; Miss J.L. Copas, Miss P. Boyle and Mr R.D. Elliott for technical assistance and Dr A. Bennett for some suggested alterations to the text.

## References

CASH, W.D., PETRACK, B. & WEINER, M. (1971). Pharmaceutical compositions which are suitable for the treatment of afflictions which manifest themselves in involuntary movements. *Ger. Offen.*, 2, 103, 284.

COLLIER, H.O.J., DINNEEN, L.C., JOHNSON, C.A. & SCHNEIDER, C. (1968). The abdominal constriction response and its suppression by analgesic drugs in the mouse. *Br. J. Pharmac.*, **32**, 295–310.

COLLIER, H.O.J., McDONALD-GIBSON, W.J. & SAEED, S.A. (1974). Apomorphine and morphine stimulate prostaglandin biosynthesis. *Nature, Lond.*, **252**, 56–58.

COLLIER, H.O.J., SAEED, S.A., SCHNEIDER, C. & WARREN, B.T. (1973). Arachidonic acid and the analgesic action of aspirin-like drugs. *Advances in the Biosciences*, **9**, 413–418.

FERREIRA, S.H., MONCADA, S. & VANE, J.R. (1971). Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature, New Biol.*, **231**, 237–239.

FINNEY, D.J., LATSCHA, R., BENNETT, B.M. & HSU, P. (1963). *Tables for Testing Significance in a 2×2 Contingency Table.* Cambridge: University Press.

FLOWER, R.J., GRYGLEWSKI, J., HERBACZYNSKA-CEDRO, & VANE, J.R. (1972). Effects on anti-inflammatory drugs on prostaglandin biosynthesis. *Nature, New Biol.*, **238**, 104–106.

GIESE, A.G., CHRISTENSEN, E. & JEPPSON, J. (1950). Absorption spectra of some sunscreens for sunburn preparations. *J. Am. Pharm. Assoc.* (Scientific Edition), **39**, 30–36.

GREENGARD, P. & PETRACK, B. (1972). Vasoconstricting and bronchodilating compositions and methods utilising gallic acid and alkyl gallates. United States Patent. 3,639,622. Assigned to Geigy Chemical Corporation.

GRIGORIU, D. (1972). Etude comparative de l'action de l'acide p-butoxyphenyl-acethydroxamique et du 17-valerate de bethametasone en application locale dans diverses dermatoses inflammatoires. *Med. et Hyg.*, **1004**, 491–492.

GRUNWALDT, E. (1972). Method for treating glaucoma with alkyl gallates. United States Patent. 3,679,804. Assigned to Geigy Chemical Corporation.

HENDERSHOT, L.C. & FORSAITH, J. (1959). Antagonism of the frequency of phenylquinone-induced writhing in the mouse by weak analgesics and non-analgesics. *J. Pharmac. exp. Ther.*, **125**, 237–240.

JELENKO, C. & WHEELER, M.L. (1972). Effects of antioxidants on the long-term utility of ethyl linoleate as a water-holding lipid for topical use postburn. *J. surg. Res.*, **12**, 161–163.

KAHN, G. & CURRY, M.C. (1974). Ultra-violent light protection by several new compounds. *Archs. Derm.*, **109**, 510–517.

KESTEN, B.M. (1956). The effects of sunlight on the skin. *J. Am. med. Assoc.*, **161**, 1565–1567.

KNOX, J.M., GUIN, J. & COCKERELL, E.G. (1957). Benzophenones. Ultra-violet light-adsorbing agents. *J. Invest. Derm.*, **29**, 435–444.

LEWIS, A.J. (1976). The assessment of systemically and topically administered anti-inflammatory drugs using u.v. erythema production in the rat. *Br. J. Pharmac.*, **56**, 385P.

McDONALD-GIBSON, W.J. & SCHNEIDER, C. (1974). A novel method for evaluating anti-inflammatory drugs in the conscious guinea-pig. *Br. J. Pharmac.*, **52**, 149P.

MODAK, A.T. & RAO, M.R.R. (1971). Propyl gallate as a local anaesthetic agent. *Indian J. med. Res.*, **59**, 795–798.

NUGTEREN, D.H., BEERTHUIS, R.K. & VAN DORP, D.A. (1966). The enzymic conversion of all-cis 8, 11, 14-eicosatrieninoic acid into prostaglandin $E_1$. *Receuil.*, **85**, 405–410.

OTOMO, S. & FUJIHIRA, E. (1970). Stabilising effect on anti-inflammatory drugs on erythrocyte membrane. I. On hemolysis and lipid peroxidation induced by $H_2O_2$ in canine erythrocyte. *J. Pharm. Soc. Japan*, **90**, 1347–1362.

POSATI, L.P., FOX, K.K. & PALLANSCH, M.J. (1970). Inhibition of bradykinin by gallates. *J. agr. Fd. Chem.*, **18**, 632–635.

SAEED, S.A., SCHNEIDER, C. & PHILLIPS, B.M. (1974). A method of treating inflammation utilising alkyl esters of gallic acid. U.S. Patent 3,833,732. Assigned to Miles Laboratories Inc.

SMITH, J.B. & WILLIS, A.L. (1971). Aspirin selectivity inhibits prostaglandin production in human platelets. *Nature New Biol.*, **231**, 235–237.

SNYDER, D.S. & EAGLSTEIN, W.H. (1974). Intradermal anti-prostaglandin agents and sunburn. *J. invest. Derm.*, **62**, 47–50.

TAPPEL, A.L., LUNDBERG, N.O. & BAYER, P.D. (1953). Effect of temperature and antioxidants upon the lipoxidase-catalysed oxidation of sodium linoleate. *Arch. Biochem. Biophys.*, **42**, 293–304.

TONELLI, G., THIBAULT, L. & RINGLER, I. (1965). A bioassay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied corticoids. *Endocrinology*, **77**, 625–634.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature, New Biol.*, **231**, 237–239.

*(Received April 8, 1976.*
*Revised June 11, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 583–592

# ANTAGONISM BY SOME ANTIHISTAMINES OF THE AMINO ACID-EVOKED RESPONSES RECORDED FROM THE LOBSTER MUSCLE FIBRE AND THE FROG SPINAL CORD

A. CONSTANTI
Department of Pharmacology, The School of Pharmacy,
University of London, 29/39 Brunswick Square, London WC1N 1AX
A. NISTRI[1]
Department of Pharmacology, St Bartholomew's Hospital Medical College,
University of London, London EC1M 6BQ

1 The effects of some antihistamines on the lobster muscle fibre and the frog spinal cord were investigated using intracellular and extracellular recordings, respectively.

2 On lobster muscle, histamine $H_1$-blockers reversibly antagonized responses to bath-applied glutamate, aspartate and quisqualate but not responses to $\gamma$-aminobutyric acid (GABA). Iontophoretic glutamate potentials were also reduced. Histamine (up to 1 mM) had no effect on this preparation.

3 The $H_1$-antagonists produced a small increase in muscle membrane conductance and a slight hyperpolarization. These effects were largely unchanged in a low $Cl^-$ bathing solution. Procaine (1 mM) decreased membrane conductance and did not affect responses to GABA or glutamate.

4 The $H_2$-antagonist burimamide blocked both glutamate and GABA-evoked responses on the lobster muscle without affecting resting potential or conductance.

5 In the frog cord, bath-applied histamine produced ventral root depolarizations and dorsal root hyperpolarizations (sometimes biphasic responses). These effects were reduced by tetrodotoxin (TTX) but not by antazoline ($H_1$-blocker) or burimamide; the latter reversibly antagonized responses to both glutamate and GABA on TTX-treated cords while antazoline was ineffective.

6 It is suggested that antihistamines can act as non-specific amino acid antagonists by interacting at the level of the receptor-coupled ionophores.

## Introduction

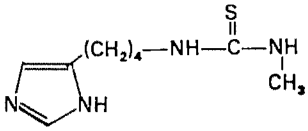
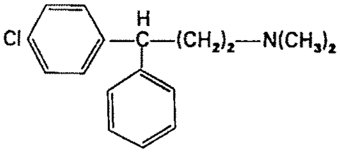
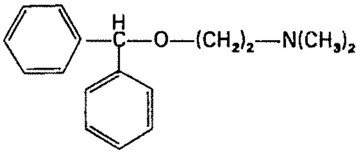
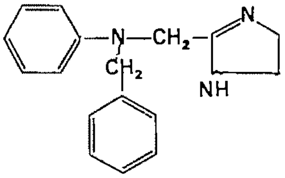
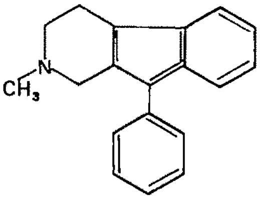
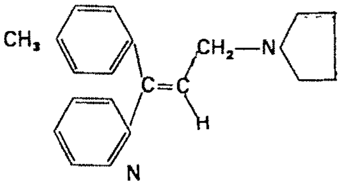
Histamine is a putative neurotransmitter in some areas of the mammalian brain (Green, 1970; Snyder & Taylor, 1972; Schwartz, 1975; Calcutt, 1976). Microiontophoretically applied histamine depresses the firing of feline brain and spinal neurones (Krnjević & Phillis, 1963; Phillis, Tebēcis & York, 1968a,b; Haas, Anders & Hosli, 1973); however, these effects are often non-specifically antagonized by histamine $H_1$- and $H_2$-receptor blockers (Phillis *et al.*, 1968a; Haas & Bucher, 1975; Haas, Wolf & Nussbaumer, 1975). This non-specificity has been tentatively attributed either to a blockade of axonal conduction (i.e. a local anaesthetic effect; Reuse, 1948; Phillis *et al.*, 1968a) or to a direct 'non-selective' action on the neuronal membrane (Haas, 1974); furthermore the ability of the antihistamines to block receptors other than the histamine receptor is well known (Goodman & Gilman, 1975).

In order to obtain more direct information on the non-specific membrane actions of the antihistamine drugs, we carried out a comparative study on two *in vitro* preparations, the lobster muscle fibre and the tetrodotoxin (TTX)-treated isolated spinal cord of the frog. The invertebrate tissue allowed a study of antihistamine action on resting membrane conductance using intracellular recordings; the experiments on the TTX-treated frog cord enabled the effect of antihistamines on responses to histamine to be investigated without possible interference from the local anaesthetic action of these compounds. Since L-glutamate and $\gamma$-aminobutyric acid (GABA) are putative neurotransmitters in both tissues

---

[1] Present address: Institute of Pharmacology, University of Florence, Viale Morgagni 65, 50134 Florence, Italy.

**Table 1** Comparison of the potency of some antihistamines in antagonizing the depolarizing action of glutamate and in increasing the resting membrane conductance of lobster muscle fibre

| *Drug* | *Chemical formula* | *Concentration (μM)* | *% Block of 100 μM glutamate response* | *% Increase in membrane conductance* |
|---|---|---|---|---|
| Burimamide |  | 40 | 0 | 0 |
| | | 100 | 7 | *0* |
| Chlorpheniramine |  | 40 | 19 | *0* |
| | | 100 | 36 | *5* |
| Diphenhydramine |  | 40 | 38 | *2* |
| | | 100 | 48 | *4* |
| Antazoline |  | 40 | 41 | *0* |
| | | 100 | 85 | 2 |
| Phenindamine |  | 40 | 58 | *7* |
| Triprolidine |  | 40 | 71 | *5* |

(Gerschenfeld, 1973; Barker, Nicoll & Padjen, 1975; Onodera & Takeuchi, 1975; Nicoll, Padjen & Barker, 1976) a quantitative assessment of the action of antihistamines on two different populations of amino acid receptors was also possible.

Our results suggest that antihistamines can increase the resting membrane conductance and act as non-specific amino acid antagonists possibly by interacting at the level of the receptor-coupled ionophores.

## Methods

Full details regarding the set-up of the lobster muscle and frog spinal cord preparations have recently been given (Constanti & Nistri, 1976a); therefore, only relevant descriptions of the methods will be presented here.

### *Lobster muscle fibre*

The opener muscle of the first or second walking leg was superfused continuously with lobster saline solution of the following composition (mM): NaCl 522, KCl 12, $CaCl_2$ 21, $MgCl_2 6H_2O$ 5, and Tris maleate 10, adjusted to pH 7.6 with 0.1 N NaOH. The membrane conductance of single superficial muscle fibres was measured by the method of Takeuchi & Takeuchi (1967) using three intracellular glass microelectrodes; one microelectrode filled with 0.6 M $K_2SO_4$ was inserted at the centre of the fibre in order to pass hyperpolarizing current pulses (0.25 Hz; 800 ms); the other two microelectrodes filled with 1.5 M K-citrate were inserted at the centre and at the end of the fibre, respectively, in order to record the resulting electrotonic potentials. GABA-evoked conductance changes (expressed as $\Delta g_m L$, where $g_m$ is the conductance per unit length and L the half-length of the fibre) were calculated with respect to the resting conductance measured immediately before the application of GABA. To allow pooling of data from different fibres, the GABA responses in each experiment were normalized with respect to the 40 μM response. Membrane depolarizations evoked by bath-applied glutamate (expressed as $\Delta V$; mV) were recorded at the centre of the muscle fibre. The current/voltage relation of the lobster fibre membrane under the present conditions was sufficiently linear in both depolarizing and hyperpolarizing directions (for potential changes $\leqslant 20$ mV) to allow depolarization responses to be taken as a quantitative measure of glutamate-receptor activation. Iontophoretic glutamate potentials were obtained by applying glutamate to single sensitive spots on the fibre membrane. Negative current pulses were passed through a microelectrode filled with 1 M L-Na glutamate (pH = 8 with NaOH; Takeuchi & Takeuchi, 1964). The retaining current was +20 nA. Iontophoretic potentials were recorded by an intracellular electrode placed about 500 μm away from the sensitive spot and photographed from the oscilloscope screen with a Polaroid camera.

### *Frog spinal cord*

The spinal cord was hemisected and placed in a small bath containing a salt solution of the following composition (mM) NaCl 109, KCl 4, $CaCl_2$ 1.5, $NaHCO_3$ 1.27, and glucose 4, pH 7.2 gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 13°C. Freshly prepared solutions of the substances to be tested were applied via the bathing medium. Recordings were made with extracellular Ag/AgCl electrodes (resistance 5–10 KΩ) from the VIIIth or IXth pairs of roots gently pulled into paraffin-filled side-chambers. Two types of potential were recorded: the ventral root potential (D-VRP) evoked by orthodromic stimulation of the adjacent dorsal root and the dorsal root potential (V-DRP) evoked by antidromic stimulation of the adjacent ventral root. Root potentials were displayed on a storage oscilloscope and a chart recorder.

### *Drugs*

The following $H_1$-receptor antagonists were used: diphenhydramine hydrochloride (Parke-Davis), antazoline hydrochloride (Ciba Labs), chlorpheniramine maleate (Allen & Hanbury), phenindamine tartrate (Roche) and triprolidine hydrochloride (Wellcome). Burimamide was used as an $H_2$-antagonist (Black, Duncan, Durant, Ganellin & Parsons, 1972). All other substances were of reagent grade. All drug solutions were adjusted to the pH of the physiological solutions.

## Results

### *Lobster muscle fibre*

*Effect of antihistamine drugs on bath-applied glutamate responses* All the antihistamine compounds tested (concentration range 40 μM to 1 mM) reversibly depressed the depolarizations produced by bath-applied glutamate. The relative potencies were assessed by measuring the % reduction of the response to 0.1 mM glutamate applied for 2 minutes. This dose gave closely reproducible responses with little desensitization and a minimal tendency to evoke a contraction of the fibre (see Constanti & Nistri, 1976b). The results of an experiment in which 6 different antihistamines were tested on the same muscle fibre is shown in Table 1. Similar studies carried out on 5 other preparations

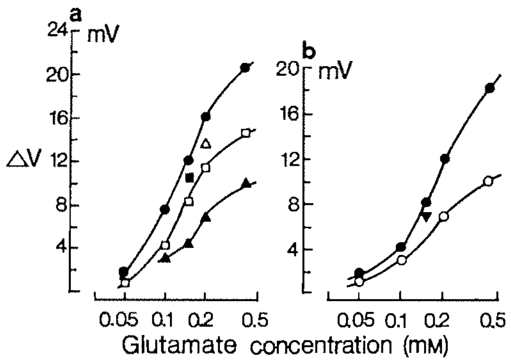


**Figure 1** (a) Effect of antazoline on the dose-response curve to glutamate recorded from a single lobster muscle fibre: (●), glutamate-evoked membrane depolarization measured in control solution; (□), in 40 μM antazoline; (▲), in 100 μM antazoline. (■), and (△) represent recovery responses recorded 10 min after return from 40 μM and 100 μM antazoline respectively. (b) Effect of burimamide on the dose-response curve to glutamate (recorded from a different fibre from that in (a)). (●), Control glutamate curve measured in normal solution; (○), in 0.5 mM burimamide; (▼), recovery response recorded 10 min after return to normal solution. Ordinates represent depolarization (mV); abscissae give the concentration of glutamate added to the superfusing solution.

confirmed this order of activity. Triprolidine was the most potent antagonist of glutamate, and burimamide the least potent. Both $H_1$-blockers and burimamide also reduced the depolarizations evoked by L-aspartate (1 to 2 mM) or by quisqualic acid (1 to 6 μM), a powerful glutamate agonist (Shinozaki & Shibuya, 1974). However, the depolarizing responses produced by doubling the KCl content of the bathing solution were unaffected.

Histamine (1 mM) had no significant effect on membrane potential or conductance, nor did it antagonize the actions of bath-applied glutamate (0.1 mM) or GABA (40 μM) (cf. McGeer, McGeer & McLennan, 1961). This result contrasts with the action of the histamine metabolite, imidazoleacetic acid which increases lobster muscle membrane conductance (Constanti & Quilliam, 1974). The antihistamine drugs (up to 1 mM) also increased the resting membrane conductance (up to 10%; cf. Table 1 and Figure 4) often accompanied by a small membrane hyperpolarization. Triprolidine and phenindamine were particularly active in this respect although in comparison with GABA (10 μM) the effect of these compounds on the conductance was notably slow in onset and decline. At higher concentrations their effect on the conductance became irreversible. Burimamide was the exception in that it did not affect resting conductance or membrane potential even at a concentration of 2 mM. The glutamate-inhibitory action of the antihistamines was not merely due to their action on the membrane conductance since such a change of conductance was insufficient to account for the total reduction in glutamate responses. Moreover, there appeared to be no relation between the ability of a compound to antagonize glutamate and its ability to increase the resting conductance (Table 1). Of the present series, antazoline combined a high glutamate inhibitory action with a low effect on the conductance; this compound was thus selected for further study.

Figure 1a shows the effect of antazoline on the glutamate dose-response curve recorded in a single lobster muscle fibre. With a progressive increase in antazoline concentration there was a slight lateral shift of the curve to the right accompanied by a depression of the apparent maximum in a seemingly non-competitive manner. Recovery of the glutamate-evoked responses was obtained within 15 min of a return to the normal solution. A reversible depression of the glutamate dose-response curve was also seen in the presence of the weaker antagonist burimamide (Figure 1b).

At the frog neuromuscular junction the local anaesthetic compound, procaine, depresses the response of the end-plate to acetylcholine by a postsynaptic blocking mechanism (Katz & Miledi, 1975). As the antihistamine drugs are also known to possess local anaesthetic activity (Reuse, 1948) it was of interest to test whether the observed antagonism of the effects of glutamate by antihistamines on lobster muscle could be mimicked by procaine. In the presence of this compound (1 mM) there was no significant inhibition of glutamate-evoked responses and the resting membrane conductance was decreased by up to 5% (see also Fatt & Katz, 1953).

*Effect on iontophoretic glutamate potential* When the glutamate-filled microelectrode was placed over a sensitive spot on the fibre membrane and a 20 ms negative current pulse passed through it (pulse frequency 0.1 Hz to avoid desensitization), a brief depolarization (i.e. the iontophoretic glutamate potential) was recorded by the nearby intracellular electrode (Figure 2a). The amplitude and the time-course of this potential were comparable to those described by Takeuchi & Takeuchi (1964) and Onodera & Takeuchi (1975) in crayfish muscle, although the latter preparation is known to be more sensitive to iontophoretically applied glutamate (Ozeki, Freeman & Grundfest, 1966). After the bath solution had been changed to one containing 0.1 mM antazoline, the amplitude of the glutamate potential was markedly reduced (Figure 2b). This effect was fully reversible upon return to a normal solution (Figure 2c) indicating that the glutamate-containing electrode had not been displaced during the

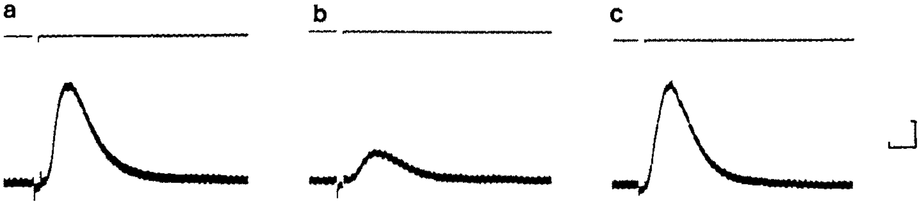


Figure 2 Effect of antazoline (100 μM) on the glutamate-evoked potential (lower beam) produced by the iontophoretic application of glutamate to a single sensitive site on the lobster fibre membrane. Upper beam shows the monitored glutamate ejection current. (a), Control glutamate-potential recorded in normal solution; (b) 2 min after changing to solution containing 100 μM antazoline; (c), recovery potential recorded 10 min after return to normal solution (superfusion system was operating throughout the experiment). Calibration: $2 \times 10^{-7}$A (upper beam), 0.5 mV (lower beam); 100 milliseconds.

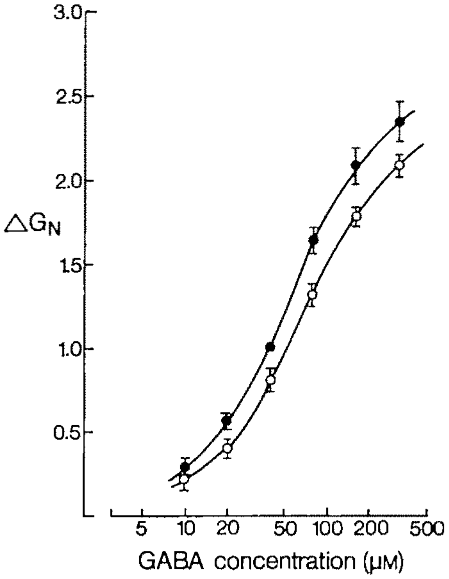


Figure 3 Effect of burimamide on the normalized γ-aminobutyric acid (GABA) log dose-conductance curve. (●), GABA-evoked increase in the membrane conductance of a lobster muscle fibre measured in normal solution; (O), in 1 mM burimamide. Ordinate scale represents normalized conductance increase ($\Delta G_N$); abscissa scale gives the concentration of GABA added to the superfusing solution. $\Delta G_N$ was calculated with respect to the 40 μM response. Points represent mean of 3 measurements made on different fibres. Vertical lines show s.e. mean.

experiment. The resting conductance and resting potential were not affected by 0.1 mM antazoline in this preparation.

*Effect on GABA dose-conductance curve* All the $H_1$-receptor blocking drugs (up to 1 mM) failed to modify the GABA dose-conductance curve. However, burimamide (0.5 to 2 mM) showed a weak antagonistic effect towards GABA. Figure 3 shows the normalized log dose-conductance curve for GABA in control solution and in the presence of 1 mM burimamide. There was a slight shift of the curve to the right along the abscissa scale which was fully reversed upon returning to control solution. A similar shift of the dose-conductance curve for imidazoleacetic acid (Constanti & Quilliam, 1974) was also observed in the presence of burimamide (not shown).

*Effect of $Cl^-$ removal on the antihistamine-evoked conductance increase* The GABA-evoked conductance change in crustacean muscle is known to be $Cl^-$-mediated (Boistel & Fatt, 1958). In order to examine whether the antihistamine-induced conductance increase also involved a $Cl^-$ permeability change, some experiments were carried out in a low $Cl^-$-bathing solution (586 mM $Cl^-$ reduced to 64 mM by replacement with Na isethionate); the changeover was first made to an intermediate low $Cl^-$-isethionate solution (586 mM $Cl^-$ reduced to 325 mM) and then after 15 min to the lower $Cl^-$-containing solution. In the experiment illustrated in Figure 4, GABA (10 μM) in control solution, produced a rapidly developing conductance change with little effect on resting potential (Figure 4a). However, antazoline (1 mM) evoked a slowly developing conductance increase accompanied by a small hyperpolarization of about 1 mV (Figure 4b). After 40 min in low $Cl^-$ (64 mM) medium, GABA depolarized the membrane (Figure 4c) in accordance with the positive shift in $Cl^-$ equilibrium potential; the hyperpolarization produced by antazoline was slightly increased (Figure 4d). Twenty min after return to a normal solution GABA hyperpolarized the membrane (suggesting some loss of intracellular $Cl^-$ during low $Cl^-$ exposure; Figure 4e) whereas the response to antazoline was similar to control (compare Figures 4b and f). Similar results were obtained with high concentrations of the other $H_1$-antihistamine drugs. These findings suggest that the antihistamine-induced conductance increase was not dependent upon $Cl^-$ and was therefore unlikely to involve an interaction with GABA receptors. The latter conclusion was supported by the finding that in

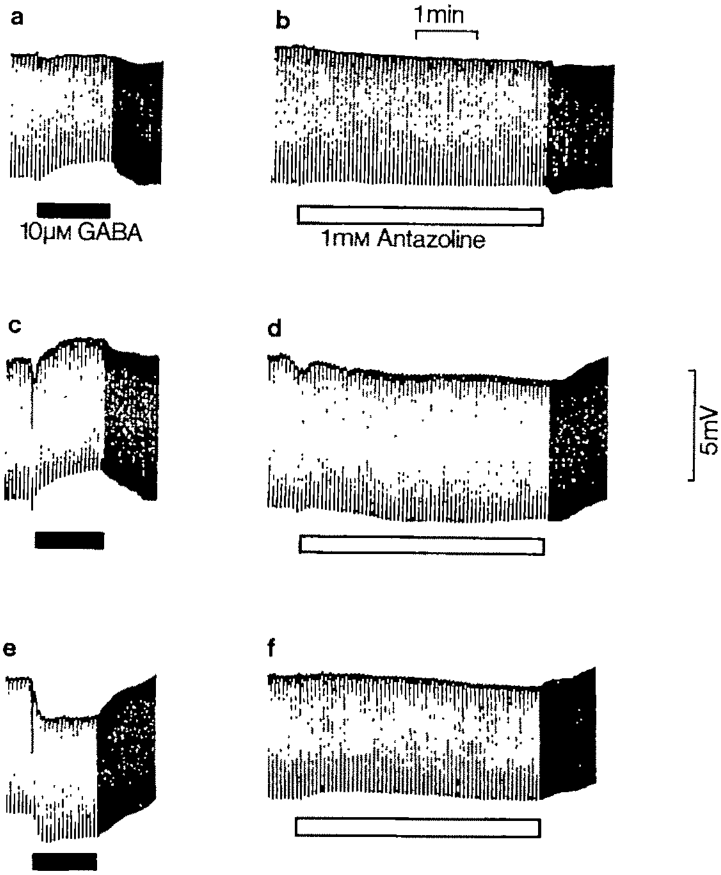


Figure 4 Effect on the membrane potential and conductance changes produced by $\gamma$-aminobutyric acid (GABA, 10 μM, filled bars) and antazoline (1 mM, open bars) on changing from a normal external solution (586 mM $Cl^-$) to a low- $Cl^-$ (64 mM) solution ($Cl^-$ replaced with isethionate- see text). Records show hyperpolarizing electrotonic potentials (downward deflections) recorded at the centre of a single lobster fibre in response to centrally applied intracellular current pulses (0.25 Hz; 800 ms; $1.6 \times 10^{-7}$A). (a) and (b) Control responses to GABA and antazoline, respectively, measured in normal solution. Note the slow onset/decline of action of antazoline and the accompanying small hyperpolarization (chart-speed was reduced by 1/3 during decline of responses); (c) and (d) 40 and 45 min respectively after changing to a low-$Cl^-$ solution. GABA now depolarized the membrane whereas antazoline produced a slightly larger hyperpolarization relative to control (b); (e) and (f) 20 and 25 min, respectively, after returning to a normal bathing solution. GABA now hyperpolarized the membrane, whereas the antazoline response was similar to control (b).

the presence of picrotoxin, a potent GABA receptor antagonist (1 μM; see Constanti & Quilliam, 1974; Nistri, Constanti & Quilliam, 1974), the antihistamine-induced conductance change was unaffected.

### *Frog spinal cord*

*Effects of histamine on root potentials* Histamine (3 mM) produced a slowly developing depolarization (0.5 to 0.7 mV) of the ventral root (onset 30 to 45 s after bath application) associated with an initial increase and subsequent decrease of the D-VRPs (Figure 5a-c); recovery was obtained after 20 min washing (Figure 5d). Histamine (3 mM) also slightly hyperpolarized the dorsal root (0.2 to 0.4 mV) and increased the V-DRPs (Figure 5f); this effect also had a slow onset and was reversed only after prolonged washing (Figure 5g). Occasionally, histamine produced biphasic responses (hyperpolarizations followed by depolarizations) of the dorsal roots. A noticeable property of the action of histamine on spinal root potentials was the tachyphylaxis which appeared after 3 or 4 applications of this substance.

In order to eliminate indirect actions of histamine due to propagated interneuronal activity, the spinal

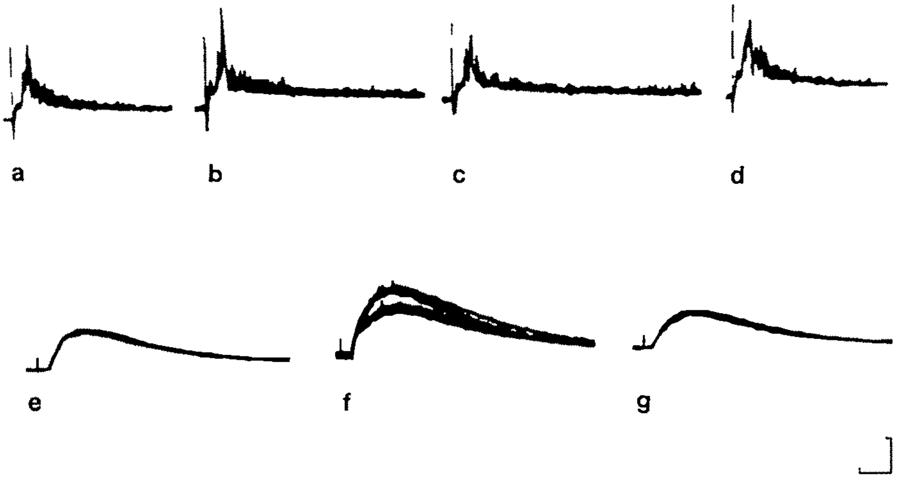


**Figure 5** Effects of histamine on D-VRPs and V-DRPs of the frog spinal cord. Stimulation parameters for all the recordings (3 superimposed traces) were: 1 Hz; 0.1 ms; supramaximal voltage. Calibration bars: 0.5 mV (negativity upwards); 20 milliseconds. (a), control D-VRPs; (b) and (c) 50 s and 60 s, respectively, after the application of histamine (3 mM); (d), recovery after 20 min washing; (e), control V-DRPs; (f), 2 min after applying histamine (3 mM), note the increased size of the V-DRPs; (g), recovery after 22 min washing.

cord was bathed with TTX (3.1 µM; see also Evans & Watkins, 1975). In the presence of TTX, D-VRPs and V-DRPs were abolished; the drug-induced responses were thus seen as changes in the d.c. level of root polarization and represented the electrotonic spreading of the potential changes of motoneurones (for ventral root recordings) or of primary afferent terminals (for dorsal root recordings). In the presence of TTX, histamine-induced responses had the same polarity as those before the application of the toxin but were reduced in size by 50 to 60%. Antazoline (0.1 mM for 30 min), burimamide (0.5 mM for 30 min) or picrotoxin (10 µM for 30 min) did not affect these dorsal or ventral root responses to histamine, irrespective of whether TTX was present. Features of the action of histamine on the cord were, therefore: a slow onset and decline rate, tachyphylaxis, weak indirect actions mainly via interneurones and insensitivity to $H_1$- or $H_2$-blockers.

*Effects of antazoline and burimamide on glutamate-evoked ventral root responses* In the presence of TTX, dose-depolarization curves for glutamate, a putative excitatory transmitter of the afferents to motoneurone somata, were obtained by recording from a lumbar ventral root (cf. Constanti & Nistri, 1976b). Figure 6a shows that antazoline (0.1 mM for 30 min) had little effect on the glutamate dose-depolarization curve. Owing to the tissue desensitization following high doses of glutamate (Constanti & Nistri, 1976b), the small increase in the level of the apparent glutamate maximum in the presence of antazoline could not be reliably interpreted.

In contrast, burimamide (0.5 mM) produced a lateral shift of the glutamate curve and a reduction of the apparent maximum (Figure 6b). All these effects were reversible after washing (40–60 minutes).

*Effects of antazoline and burimamide on GABA-evoked dorsal root responses* In the presence of TTX, antazoline (0.1 mM for 30 min) did not affect the dose-depolarization curve for GABA (the putative transmitter released onto the primary afferent terminals; Barker *et al.*, 1975). However, burimamide (0.5 mM) reduced the GABA maximum and produced a lateral shift of the curve along the abscissa scale (Figure 7). Burimamide also depressed the dorsal root responses evoked by taurine (3 mM); thus, the blocking action of burimamide resembled that of picrotoxin on this preparation (Constanti & Nistri, 1976a; Nistri & Constanti, 1976).

## Discussion

On lobster muscle, all the $H_1$-antagonists tested shifted the glutamate dose-depolarization curve in an apparently non-competitive manner but did not affect the GABA dose-conductance curve. These agents also produced a small increase in membrane conductance that was insensitive to picrotoxin and a membrane hyperpolarization relatively unaffected by a low $Cl^-$ bathing solution. This effect was probably due to an increase in the passive ionic permeability of the membrane, possibly to $K^+$. In contrast, the $H_2$-antagonist burimamide was a weak antagonist of both

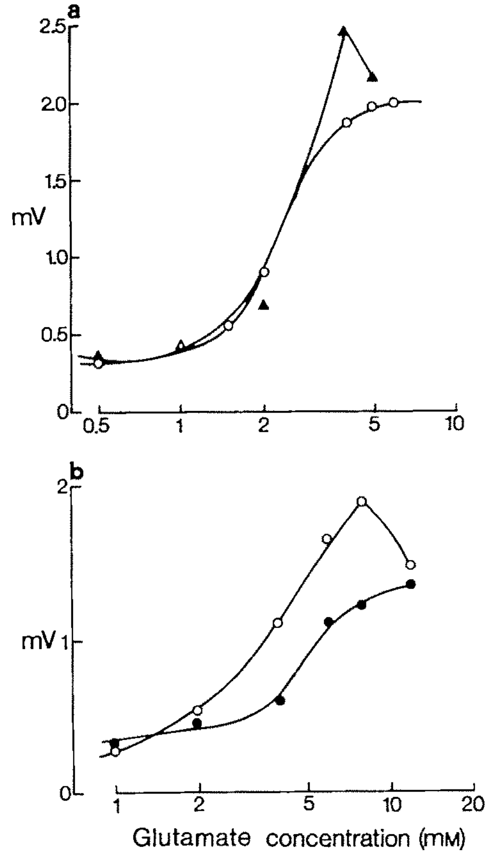


**Figure 6** Effect of antihistamines on the glutamate dose-depolarization curve recorded from the frog spinal cord. Ventral root recordings in the presence of tetrodotoxin (TTX, 3.1 μM). (a) (O) Control glutamate curve; (▲), in the presence of antazoline (100 μM); (b) (O), control glutamate curve; (●), in the presence of burimamide (500 μM). Note that (a) and (b) were obtained from two different cords.

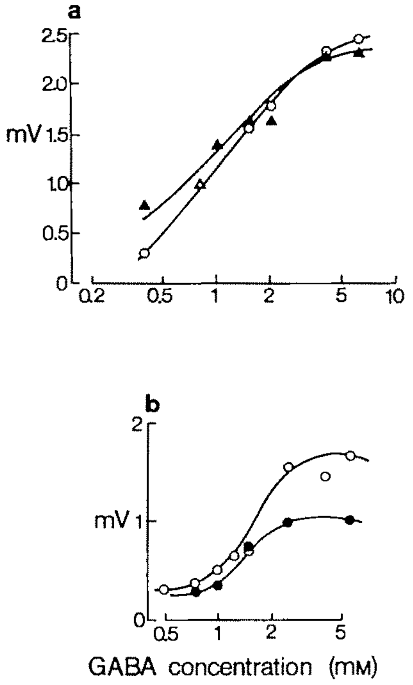


**Figure 7** Effect of antihistamines on the $\gamma$-aminobutyric acid (GABA) dose-depolarization curve recorded from the frog spinal cord. Dorsal root recordings in the presence of tetrodotoxin (TTX, 3.1 μM). (a) (O), Control GABA curve; (▲), in the presence of antazoline (100 μM); (b) (O), Control GABA curve; (●), in the presence of burimamide (500 μM). Note that (a) and (b) were obtained from two different cords.

glutamate and GABA responses and did not affect the resting conductance. We cannot consider $H_1$-blockers as specific glutamate antagonists on lobster muscle since the effect of aspartate was also reduced; moreover, the lack of selectivity of burimamide towards glutamate and GABA responses indicated that this compound was a non-selective amino acid antagonist on this preparation.

On the frog spinal cord, histamine produced small and variable changes in the root potentials. The characteristic features for the action of histamine were: a slow onset and decline in rate of action compared to glutamate and GABA (see Constanti & Nistri, 1976a,b) and the occurrence of tachyphylaxis. Since the effects of histamine were mainly indirect and not antagonized by antazoline or burimamide it seemed unlikely that histamine had an important role in the transmission processes investigated. In the presence of TTX, a very potent local anaesthetic agent, antazoline (the most powerful glutamate antagonist on lobster muscle with a minimal effect on resting conductance) did not significantly affect the glutamate or GABA dose-depolarization curves whereas burimamide depressed both curves in a seemingly non-competitive manner.

It is possible that the $H_1$-antagonists on lobster muscle and burimamide on the frog spinal cord were interfering with amino acid responses by interacting with receptor-coupled ionophores (or with the coupling mechanism between receptor and ionophore); however, an additional small action on glutamate receptor binding sites cannot be excluded. The effect of these antihistamine agents on the receptor-coupled ionophores would explain the apparent non-competitive depression of the glutamate and GABA dose-response curve. Since glutamate and GABA responses on crustacean muscle are largely mediated by $Na^+$ and $Cl^-$ respectively (Takeuchi & Takeuchi, 1967; Onodera & Takeuchi, 1975;

Constanti & Nistri, 1976b), the $H_1$-antagonists might block glutamate-activated $Na^+$ channels in the lobster muscle membrane, while burimamide might be a weaker and rather non-selective channel-blocking compound. A similar proposal was made by Barker & Gainer (1973) and Barker (1975a,b) to explain the action of pentobarbitone on lobster and crayfish muscles. Like the $H_1$-antihistamines on lobster muscle, pentobarbitone depressed the glutamate dose-depolarization curve in a seemingly non-competitive manner without effect on GABA responses. Barker (1975a) suggested that inhibition of the glutamate responses was dependent on the hydrophobic nature of the barbiturate molecule rather than on some steric factor. A similar proposal may be applicable to the antihistamines; however, the exact mechanism by which all these agents produce their effect has yet to be determined.

Blockade of receptor-coupled $Na^+$ conductance would also explain the action of burimamide on the frog spinal cord where the effects of glutamate and GABA seem to involve in part an increase in membrane $Na^+$ permeability (Barker & Nicoll, 1973; Constanti & Nistri, 1976a,b). The failure of antazoline (0.1 mM) to antagonize glutamate responses in the frog cord compared with the result of the experiments with lobster muscle remains unclear. Perhaps the glutamate-activated $Na^+$ channels of amphibian motoneurones are different in nature from those of the lobster muscle.

In view of the present findings, care must be exercised in the use of $H_1$-receptor blockers to study effects of histamine in the central nervous system. The antagonistic actions of these compounds towards amino acid-evoked responses and their ability to increase resting membrane conductance should be taken into consideration.

We wish to thank Miss Sheila Harper and Mr J.C.R. Gomersall for skilful technical assistance. We also thank Prof. J.W. Black (University College London) for the kind gift of burimamide, Prof. T. Takemoto (Tohoku University, Sendai, Japan) for the gift of quisqualic acid and the pharmaceutical companies which generously donated the antihistamine drugs used in this study.

## References

BARKER, J.L. (1975a). CNS depressants: effects on post-synaptic pharmacology. *Brain Res.*, **92**, 33–55.

BARKER, J.L. (1975b). Inhibitory and excitatory effects of CNS depressants on invertebrate synapses. *Brain Res.*, **93**, 77–90.

BARKER, J.L. & GAINER, H. (1973). Pentobarbital: selective depression of excitatory post-synaptic potentials. *Science*, **182**, 720–722.

BARKER, J.L. & NICOLL, R.A. (1973). The pharmacology and ionic dependency of amino acid responses in the frog spinal cord. *J. Physiol., Lond.*, **228**, 259–277.

BARKER, J.L., NICOLL, R.A. & PADJEN, A. (1975). Studies on convulsants in the isolated frog spinal cord. II. Effects on root potentials. *J. Physiol., Lond.*, **245**, 537–548.

BLACK, J.W., DUNCAN, W.A.M., DURANT, C.J., GANELLIN, C.R. & PARSONS, E.M. (1972). Definition and antagonism of histamine $H_2$-receptors. *Nature, Lond.*, **236**, 385–390.

BOISTEL, J. & FATT, P. (1958). Membrane permeability change during inhibitory transmitter action in crustacean muscle. *J. Physiol., Lond.*, **144**, 176–191.

CALCUTT, C.R. (1976). The role of histamine in the brain. *Gen. Pharmac.*, **7**, 15–25.

CONSTANTI, A. & NISTRI, A. (1976a). A comparative study of the action of γ-aminobutyric acid and piperazine on the lobster muscle fibre and the frog spinal cord. *Br. J. Pharmac.* **57**, 347–358.

CONSTANTI, A. & NISTRI, A. (1976b). A comparative study of the effects of glutamate and kainate on the lobster muscle fibre and the frog spinal cord. *Br. J. Pharmac.* **57**, 359–368.

CONSTANTI, A. & QUILLIAM, J.P. (1974). A comparison of the effects of GABA and imidazoleacetic acid on the membrane conductance of lobster muscle fibre. *Brain Res.*, **79**, 306–310.

EVANS, R.H. & WATKINS, J.C. (1975). Ventral root responses of the hemisected amphibian spinal cord to perfused amino acids in the presence of procaine. *Br. J. Pharmac.*, **55**, 519–526.

FATT, P. & KATZ, B. (1953). The electrical properties of crustacean muscle fibres. *J. Physiol., Lond.*, **120**, 171–204.

GERSCHENFELD, H.M. (1973). Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. *Physiol. Rev.*, **53**, 1–119.

GOODMAN, L.S. & GILMAN, A. (1975). *The Pharmacological Basis of Therapeutics.* 5th edition pp. 603–613. New York: Macmillan.

GREEN, J.P. (1970). Histamine. In *Handbook of Neurochemistry*, ed. Lajtha, A. vol. 4, pp. 221–250. New York: Plenum Press.

HAAS, H.L. (1974). Histamine: action on single hypothalamic neurones. *Brain Res.*, **76**, 363–366.

HAAS, H.L., ANDERS, E.G. & HOSLI, L. (1973). Histamine and metabolites: their effects and interactions with convulsants on brain stem neurones. *Brain Res.*, **51**, 269–278.

HAAS, H.L. & BUCHER, U.M. (1975). Histamine $H_2$-receptors on single central neurones. *Nature, Lond.*, **255**, 634–635.

HAAS, H.L., WOLF, P. & NUSSBAUMER, J.C. (1975). Histamine: action on supraoptic and other hypothalamic neurones of the cat. *Brain Res.*, **88**, 166–170.

KATZ, B. & MILEDI, R. (1975). The effect of procaine on the action of acetylcholine at the neuromuscular junction. *J. Physiol., Lond.*, **2[illegible]0**, 269–284.

KRNJEVIĆ, K. & PHILLIS, J.W. (1963). Actions of certain amines on cerebral cortical neurones. *Br. J. Pharmac. Chemother.*, **20**, 471–490.

McGEER, E.G., McGEER, P.L. & McLENNAN, H. (1961). The inhibitory action of 3-hydroxytyramine, gamma-aminobutyric acid (GABA) and some other compounds toward the crayfish stretch receptor neuron. *J. Neurochem.*, **8**, 36–49.

NICOLL, R.A., PADJEN, A. & BARKER, J.L. (1976). Analysis of amino acid responses on frog motoneurones. *Neuropharmacology*, **15**, 45–53.

NISTRI, A. & CONSTANTI, A. (1976). The action of taurine on the lobster muscle fibre and the frog spinal cord. *Neuropharmacology*, (in press).

NISTRI, A., CONSTANTI, A. & QUILLIAM, J.P. (1974). Central inhibition, GABA and tutin. *Lancet*, **i**, 996–997.

ONODERA, K. & TAKEUCHI, A. (1975). Ionic mechanism of the excitatory synaptic membrane of the crayfish neuromuscular junction. *J. Physiol., Lond.*, **252**, 259–318.

OZEKI, M., FREEMAN, A.R. & GRUNDFEST, H. (1966). The membrane components of crustacean neuromuscular systems. II. Analysis of interactions among electrogenic components. *J. gen. Physiol.*, **49**, 1335–1349.

PHILLIS, J.W., TEBĒCIS, A.K. & YORK, D.H. (1968a). Histamine and some antihistamines: their actions on cerebral cortical neurones. *Br. J. Pharmac.*, **33**, 426–440.

PHILLIS, J.W., TEBĒCIS, A.K. & YORK, D.H. (1968b). Depression of spinal motoneurones by noradrenaline, 5-hydroxytryptamine and histamine. *Eur. J. Pharmac.*, **4**, 471–475.

REUSE, J.J. (1948). Comparisons of various histamine antagonists. *Br. J. Pharmac. Chemother.*, **3**, 174–180.

SCHWARTZ, J.C. (1975). Histamine as a transmitter in brain. *Life Sci.*, **17**, 503–518.

SHINOZAKI, H. & SHIBUYA, I. (1974). A new potent excitant, quisqualic acid: effects on crayfish neuromuscular junction. *Neuropharmacology*, **13**, 665–672.

SNYDER, S.H. & TAYLOR, K.M. (1972). Histamine in the brain: a neurotransmitter? In *Perspectives in Neuropharmacology*, ed. Snyder, S.H. pp. 43–73. New York: Oxford University Press.

TAKEUCHI, A. & TAKEUCHI, N. (1964). The effect on crayfish muscle of iontophoretically applied glutamate. *J. Physiol., Lond.*, **170**, 296–317.

TAKEUCHI, A. & TAKEUCHI, N. (1967). Anion permeability of the inhibitory post-synaptic membrane of the crayfish neuromuscular junction. *J. Physiol., Lond.*, **191**, 575–590.

*(Received April 13, 1976.*
*Revised June 18, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 593–598

# CENTRALLY INDUCED HYPOTENSION AND BRADYCARDIA AFTER ADMINISTRATION OF α-METHYLNORADRENALINE INTO THE AREA OF THE NUCLEUS TRACTUS SOLITARII OF THE RAT

W. DE JONG & F.P. NIJKAMP
Rudolf Magnus Institute for Pharmacology, University of Utrecht, Medical Faculty,
Vondellaan 6, Utrecht, The Netherlands

1 In anaesthetized rats, bilateral injections of α-methylnoradrenaline, noradrenaline or adrenaline into the area of the nucleus tractus solitarii (NTS) of the brain stem caused dose-dependent decreases of systemic arterial blood pressure and heart rate. The effects of α-methylnoradrenaline were most pronounced and lasted longest.

2 The cardiovascular effects of α-methylnoradrenaline appeared to be restricted to the medio-caudal part of the NTS.

3 Prior administration of the α-adrenoceptor blocking agent, phentolamine, reversed the fall in blood pressure and heart rate induced by α-methylnoradrenaline into an increase.

4 Systemic administration of atropine combined with vagotomy potentiated the inhibitory effects of α-methylnoradrenaline on the cardiovascular system.

## Introduction

Evidence is accumulating for a centrally mediated blood pressure lowering effect of α-methyldopa (Van Zwieten, 1975). The metabolite which mediates the central effect on blood pressure is thought to be α-methylnoradrenaline since inhibition of central dopa-decarboxylase or dopamine-β-hydroxylase activity prevents the central hypotensive action of α-methyldopa (Henning & Rubenson, 1971; Day, Roach & Whiting, 1973; Henning, 1975; Nijkamp, Ezer & De Jong, 1975). Experiments in cats in which α-methyldopa was infused into the vertebral artery indicated that the main site of action may be located in the medulla oblongata (Henning & Van Zwieten, 1968). Further, we have shown (Nijkamp & De Jong, 1975), that local injection of α-methylnoradrenaline into the nucleus tractus solitarii (NTS) of the medulla oblongata caused a reduction in blood pressure and heart rate. Pressor doses of the peptides angiotensin or vasopressin were without effect. Injections or infusions of α-methyldopa, α-methyldopamine or α-methylnoradrenaline into the cerebral ventricles of anaesthetized or conscious cats or rats caused hypotensive responses which were prevented by α-adrenoceptor antagonists (Heise & Kroneberg, 1972; Day & Roach, 1974; Finch, Hersom & Hicks, 1975). The present study describes the site of action of α-methylnoradrenaline in the NTS and compares this action with that of noradrenaline and adrenaline. The results obtained are described here in detail supplementing a brief preliminary report which has already been made (De Jong, Nijkamp & Bohus, 1975a).

## Methods

The experiments were performed on male Wistar rats (outbred stock, Wi/Cpb, Zeist) weighing 200–240 grams. The animals were anaesthetized with urethane (1.25 g/kg) and placed in a stereotaxic apparatus (David Kopf) with the head flexed to 45°. The dorsal surface of the lower brain stem was then exposed by a limited occipital craniotomy. The obex (corresponding with the rostral part of the area postrema) was used as a stereotaxic zero. Bilateral injections into the NTS (0.5 mm lateral to the obex and at a depth of 1.0 mm) were carried out with a stainless steel needle (outer diameter 0.2 mm) connected via a polyethylene cannula with an Agla micrometer syringe and a Shardlow micrometer. Injections in other sites in the NTS and outside the NTS were also given at a depth of 1 mm into the brain stem. Drugs were dissolved in 0.9% w/v NaCl solution (saline) and administered bilaterally in a volume of 0.6 µl over a period of 30 seconds. Control rats received 0.6 µl of saline bilaterally.

Blood pressure was continuously recorded from a permanent indwelling iliac cannula with a Statham transducer (Model P23AC) connected to a Grass polygraph. The iliac cannula had been implanted under ether anaesthesia at least 24 h before the experiment (Nijkamp *et al.*, 1975). Heart rate was computed from the blood pressure pulse wave by a cardiotachometer (Narco Bio systems, model BT1200). Bilateral vagotomy was carried out under ether anaesthesia at mid-cervical level 30 min before local injection of $\alpha$-methylnoradrenaline. In experiments in which vagotomy was performed, atropine (5 mg/kg i.p.) was given at the same time. Drugs given intravenously were dissolved in 0.2 ml saline and administered into the right jugular vein.

After termination of the experiments, the brains were fixed in 5% formalin. Frozen sections of 100 µm were cut and stained with 0.1% thionine. The injection sites were controlled microscopically.

Results are expressed as means ± standard error of the mean (s.e. mean). Significance of the difference between values of control and treated groups was determined with Student's *t*-test.

The following drugs were used: (–)-adrenaline bitartrate (Boehringer); atropine sulphate (OPG); (±)-erythro-$\alpha$-methylnoradrenaline hydrochloride (Cobrefin, Winthrop Labs.); (–)-noradrenaline bitartrate (Levarterenol, OPG); phentolamine methane sulphonate (Regitine, Ciba); urethane (OPG). Drug weights refer to the salts except for those of adrenaline, noradrenaline and $\alpha$-methylnoradrenaline.

## Results

Neither blood pressure nor heart rate changed in the different groups during the operative procedure. Basal values ranged from 99 ± 3 mmHg to 130 ± 5 mmHg and 379 ± 22 beats/min to 462 ± 11 beats/min, respectively.

Bilateral microinjection of 23 nmol (±)-$\alpha$-methylnoradrenaline into the area of the NTS at the level of the obex caused a decrease of blood pressure and heart rate (Figure 1). Blood pressure and heart rate started to fall immediately after completion of the injections and reached a maximum of −33 ± 6 mmHg after 5 min and −66 ± 7 beats/min after 10 min, respectively. After 30 min, blood pressure was still significantly different from its control value ($P < 0.05$). A representative track into the area of the NTS is indicated in a cross section of the medulla oblongata at the level of the obex (Figure 2). Data from animals in which tracks were located more laterally or more medially were not used for this group.

Unilateral injection of 23 nmol $\alpha$-methylnoradrenaline also caused a lowering in blood pressure and heart rate (maximal responses −22 ± 5 mmHg and −59 ± 15 beats/min, respectively, $n = 8$). In comparison with the responses after bilateral injections, these effects were less marked ($P < 0.05$ for the effect on blood pressure) and both blood pressure and heart rate returned to basal values within 20 minutes.

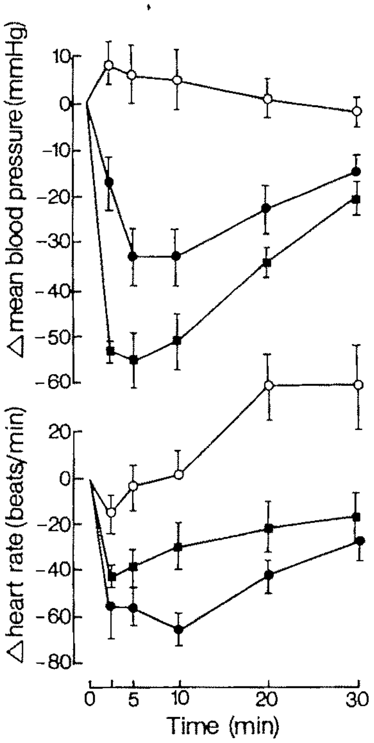


**Figure 1** Effect of bilateral injection of $\alpha$-methylnoradrenaline (23 nmol) into the area of the nucleus tractus solitarii on mean blood pressure and heart rate of anaesthetized rats and of vagotomized rats treated with atropine sulphate (5 mg/kg i.p.); (○) saline ($n = 9$); (●) $\alpha$-methylnoradrenaline ($n = 6$); (■) $\alpha$-methylnoradrenaline after vagotomy and atropine ($n = 6$).

In order to determine the effective localization in the NTS, the dose of 23 nmol $\alpha$-methylnoradrenaline was applied in sixteen different sites of the medulla oblongata (Figure 3). The maximal blood pressure lowering effect occurred just caudal to the obex level, 0.5 mm lateral to the midline (−36 ± 1 mmHg). However, between 0.5 mm rostral and 1.0 mm caudal to the obex a similar but less pronounced effect on blood pressure was observed. The fall in blood pressure was associated with decreases in heart rate. All effective sites for the inhibitory cardiovascular effects were located in the NTS.

In order to see whether adrenaline and noradrenaline also decrease blood pressure after injection into the NTS a study was undertaken to compare the cardiovascular effects of three different doses (5.8, 23, 92 nmol) of these catecholamines (Figure 4). The effects on blood pressure were dose-dependent, except for the highest dose of noradrenaline (92 nmol) which caused a maximal decrease of blood pressure of only −19 ± 7 mmHg.

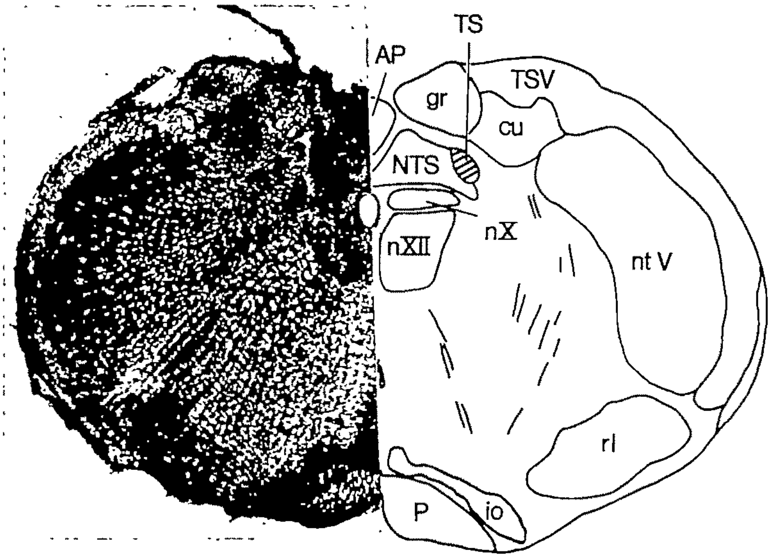


**Figure 2** Cross section of the medulla oblongata at the level of the obex with photographic (left) reflection of tracks in the area of the nucleus tractus solitarii (0.5 mm lateral, 1 mm depth). Schematic representation of structures is shown in the right half of the figure. Abbreviations are according to Palkowitz & Jacobowitz (1974): AP = area postrema; cu = nucleus cuneatus; gr = nucleus gracilis; io = nucleus olivaris inferior; NTS = nucleus tractus solitarii; rl = nucleus reticularis; ntV = nucleus tractus spinalis nervi trigemini; nX = nucleus originis dorsalis vagi; nXII = nucleus originis nervi hypoglossi; P = tractus cortico spinalis; TS = tractus solitarius; TSV = tractus spinalis nervi trigemini.

This was a significantly smaller decrease ($P < 0.05$) than that following 92 nmol α-methylnoradrenaline (−43 ± 4 mmHg) or adrenaline (−36 ± 3 mmHg). The maximal decrease in blood pressure after administration of adrenaline and noradrenaline was reached after 2.5 to 5 minutes. However, for α-methylnoradrenaline, the maximal response was not reached until 5 to 10 minutes. A similar pattern was observed for the effects on heart rate.

In order to study the effect of α-adrenoceptor blockade on the cardiovascular effects of α-methylnoradrenaline, the α-adrenoceptor antagonist, phentolamine, was injected bilaterally into the area of the NTS in a dose of 23 nmol, 10 min before the local

**Table 1** Effect of bilateral injection of α-methylnoradrenaline (23 nmol) into the area of the nucleus tractus solitarii on mean blood pressure and heart rate of anaesthetized rats. Either saline or phentolamine (23 nmol) was injected bilaterally into the same site 10 min earlier

| | | *Pretreatment* | | *Pretreatment* | |
|---|---|---|---|---|---|
| | | *Saline* | *Phentolamine* | *Saline* | *Phentolamine* |
| *Time (min) after injection of α-methylnoradrenaline* | | *Blood pressure (mmHg)* | | *Heart rate (beats/min)* | |
| −10.0 (basal values) | | 127 ± 6 (10) | 115 ± 4 (8) | 379 ± 22 | 420 ± 22 |
| 0 | | 123 ± 7 | 121 ± 7 | 357 ± 17 | 343 ± 22 |
| 1.0 | Change with respect to time 0 values | −11 ± 5 | +36 ± 6** | −39 ± 18 | +36 ± 12* |
| 2.5 | | −20 ± 4 | +28 ± 6** | −27 ± 16 | +31 ± 15* |
| 5.0 | | −33 ± 4 | +14 ± 7** | −19 ± 19 | +31 ± 19 |
| 10.0 | | −30 ± 4 | +8 ± 5** | −7 ± 24 | +43 ± 24 |
| 20.0 | | −21 ± 4 | +3 ± 7* | +23 ± 24 | +69 ± 20 |
| 30.0 | | −13 ± 4 | +4 ± 8 | +44 ± 21 | +78 ± 19 |

* $P < 0.05$; ** $P < 0.01$; compared to corresponding control value (saline pretreatment). Number of animals in parentheses.

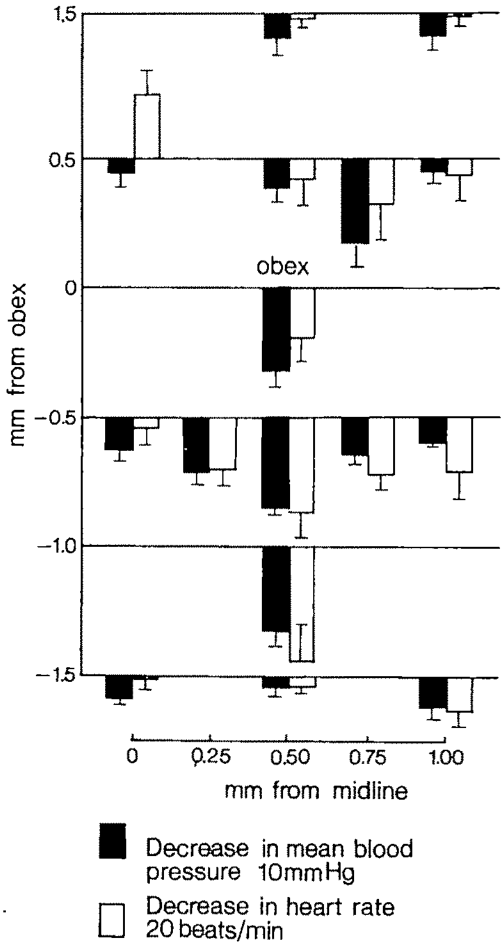


**Figure 3** Topographical distribution in the medulla oblongata of maximal mean blood pressure and heart rate responses to 23 nmol α-methylnoradrenaline injected in sixteen different sites. Except in the midline, all injections were given bilaterally, ($n = 5$ to 11).

injection of 23 nmol α-methylnoradrenaline. After phentolamine, the fall in blood pressure and heart rate was prevented and even reversed to an increase, the maximum occurring within approximately 2 min (Table 1).

Following bilateral vagotomy and intraperitoneal injection of atropine sulphate (5 mg/kg), 23 nmol α-methylnoradrenaline caused a decrease in blood pressure with a maximum of −55 mmHg at 5 min after injection (Figure 1). After 30 min, blood pressure was still significantly different ($P < 0.01$) from the group treated with saline only. The decrease in blood pressure after the parasympathetic blockade seems to be intensified and to reach its maximum effect faster. In contrast, the decrease in heart rate was reduced by 35% in the pretreated group.

## Discussion

These results confirm and extend our previous findings that α-methylnoradrenaline causes a lowering of blood pressure and heart rate after local injection into the area of the NTS (Nijkamp & De Jong, 1975; De Jong *et al.*, 1975a). Only in part of the NTS directly around the obex were these effects observed. This area of the NTS has a high density of catecholaminergic terminals as visualized histochemically (Fuxe, 1965). Mimicking of the effects of the endogenous neurotransmitter noradrenaline by α-methylnoradrenaline in this part of the brain seems a possibility, since noradrenaline also causes a fall in blood pressure after local application (De Jong, 1974; De Jong, Zandberg & Bohus, 1975b). However, α-methylnoradrenaline was more effective than noradrenaline. Because the responses to α-methylnoradrenaline were more prolonged than those to noradrenaline or adrenaline it is tempting to speculate that α-methyldopa produces its inhibitory effects on the cardiovascular system through α-methylnoradrenaline which, in the central nervous system, may be a more effective neurotransmitter on noradrenaline receptor sites. α-Adrenoceptors seem to be involved since phentolamine blocked the inhibitory effects and even reversed these effects to an initial increase in blood pressure and heart rate. This finding is in agreement with the inhibitory effect of intraventricularly injected phentolamine in rats on the blood pressure lowering effect of α-methyldopa (Finch & Haeusler, 1973). The effects on cardiovascular functions exerted in the NTS are stereospecific since (+)-noradrenaline was ineffective (De Jong & Nijkamp, 1975) and (+)-α-methylnoradrenaline in doses up to 200 nmol also failed to induce changes in blood pressure and heart rate (P. Zandberg & W. de Jong, unpublished observations). It is therefore likely that the difference in potency between (−)-α-methylnoradrenaline and (−)-noradrenaline is even greater than the difference between the racemic mixture of α-methylnoradrenaline and (−)-noradrenaline used in this study.

The increase in blood pressure and heart rate observed after α-blockade might be caused by an effect on β-adrenoceptors since α-methylnoradrenaline possesses β-agonistic activity and intraventricular injection of β-mimetics such as isoprenaline sometimes causes pressor responses and tachycardia (Toda, Matsuda & Shimamoto, 1969; Satchell, Freeman & Hopkins, 1971; Bhargava, Mishra & Tangri, 1972; Day & Roach, 1974).

After atropine and vagotomy the decrease in blood pressure after local injection of α-methylnoradrenaline into the area of the NTS is augmented. In unanaesthetized dogs, atropine antagonizes the blood pressure increase following intraventricular injection of acetylcholine into the brain (Lang & Rush, 1973; Laubie, 1975) while the hypertensive response is

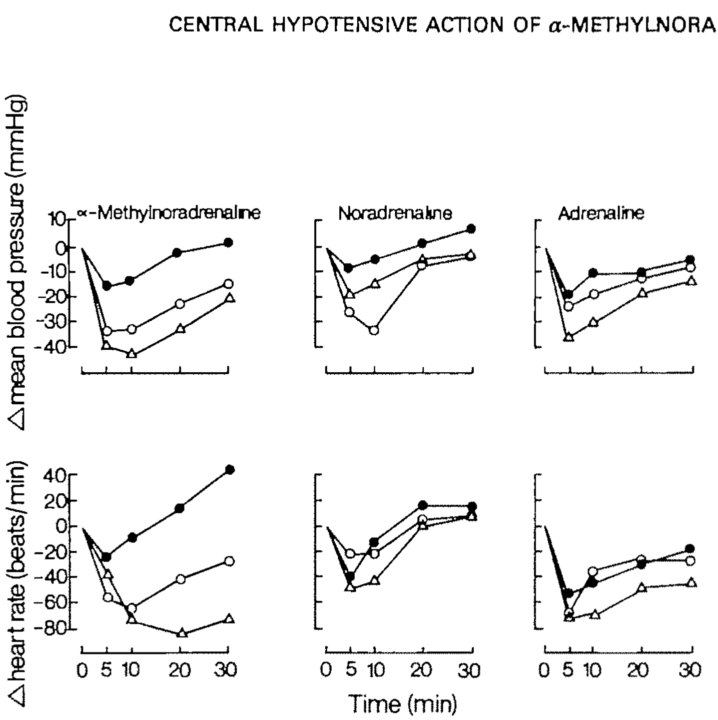


**Figure 4** Effect of different doses of α-methylnoradrenaline, noradrenaline and adrenaline on mean blood pressure and heart rate of anaesthetized rats, ($n$=4 to 9): (●) 5.8 nmol; (○) 23.0 nmol; (△) 92.0 nmol.

potentiated by physostigmine. We found a dose-dependent increase in blood pressure after local injection of 1 and 5 µg physostigmine into the area of the NTS (unpublished results). So the more pronounced decrease in blood pressure by local injection of α-methylnoradrenaline after atropine and vagotomy might be explained by an inhibition of the activation of muscarinic receptors. In fact, this inhibitory effect of the cholinergic system does not seem to be restricted to the hypotensive action of α-methylnoradrenaline since Laubie (1975) reported that in unanaesthetized dogs, stimulation of muscarinic receptors counteracted the decrease in blood pressure brought about by the centrally acting anti-hypertensive agent clonidine (Schmitt, Schmitt, Fenard & Laubie, 1973; Schmitt, 1975), while vertebral artery injection of atropine allowed the effect to reappear.

It has been shown by Palkovitz & Jacobowitz (1974) that a reasonable amount of acetylcholinesterase activity is present just under the NTS. A neurotransmitter function for acetylcholine in this part of the brain, mediating the stimulating properties of α-methylnoradrenaline, therefore, seems possible. On this basis, it cannot be excluded that the antihypertensive agent α-methyldopa acting through its metabolite α-methylnoradrenaline may exert two opposing effects; an increase of blood pressure may be caused via a cholinergic synapse as well as a decrease of blood pressure mediated via noradrenergic synapse.

We thank Dr F.C. Nachod of Sterling-Winthrop Research Institute for providing us with (±)-α-methylnoradrenaline hydrochloride (Cobrefin). The skilful assistance of Mr H. de Lang and Mr L. van Halewijn is gratefully acknowledged.

## References

BHARGAVA, K.P., MISHRA, N. & TANGRI, K.K. (1972). An analysis of central adrenoceptors for control of cardiovascular function. *Br. J. Pharmac.*, **45**, 596–602.

DAY, M.D., ROACH, A.G. & WHITING, R.L. (1973). The mechanism of the antihypertensive action of α-methyldopa in hypertensive rats. *Eur. J. Pharmac.*, **21**, 271–280.

DAY, M.D. & ROACH, A.G. (1974). Central α- and β-adrenoceptors modifying arterial blood pressure and heart rate in conscious cats. *Br. J. Pharmac.*, **51**, 325–333.

DE JONG, W. (1974). Noradrenaline: central inhibitory control of blood pressure and heart rate. *Eur. J. Pharmac.*, **29**, 179–181.

DE JONG, W. & NIJKAMP, F.P. (1975). Hypotensive action of noradrenaline and $\alpha$-methylnoradrenaline in the area of the nucleus tractus solitarii in the rat brain stem. In *Central Action of Drugs in Blood Pressure Regulation*, ed. Davies, D.S. & Reid, J.L. pp. 179–180. London: Pitman Publishing Ltd.

DE JONG, W., NIJKAMP, F.P. & BOHUS, B. (1975a). Role of noradrenaline and serotonin in the central control of blood pressure in normotensive and spontaneously hypertensive rats. *Archs Int. Pharmacodyn. Ther.*, **213**, 272–284.

DE JONG, W., ZANDBERG, P. & BOHUS, B. (1975b). Central inhibitory noradrenergic cardiovascular control. *Progress in Brain Research*, **42**, 285–298.

FINCH, L. & HAEUSLER, G. (1973). Further evidence for a central hypotensive action of $\beta$-methyldopa in both the rat and the cat. *Br. J. Pharmac.*, **47**, 217–228.

FINCH, L., HERSOM, A. & HICKS, P. (1975). Studies on the hypotensive action of $\alpha$-methyldopamine. *Br. J. Pharmac.*, **54**, 445–451.

FUXE, K. (1965). Evidence for the existence of monoamine neurons in the central nervous system. IV. The distributions of monoamine nerve terminals in the central nervous system. *Acta physiol. scand.*, **64**, Suppl. 247, 39–85.

HEISE, A. & KRONEBERG, G. (1972). $\alpha$-Sympathetic receptor stimulation in the brain and hypotensive activity of $\alpha$-methyldopa. *Eur. J. Pharmac.*, **17**, 315–317.

HENNING, M. (1975). Central sympathetic transmitters and hypertension. *Clin. Sci. Mol. Med.*, **48**, 195s–203s.

HENNING, M. & RUBENSON, R. (1971). Evidence that the hypotensive action of methyldopa is mediated by central actions of methylnoradrenaline. *J. Pharm. Pharmac.*, **23**, 407–411.

HENNING, M. & VAN ZWIETEN, P.A. (1968). Central hypotensive effect of $\alpha$-methyldopa. *J. Pharm. Pharmac.*, **20**, 409–417.

LANG, W.J. & RUSH, M.L. (1973). Cardiovascular responses to injections of cholinergic drugs into the cerebral ventricles of unanaesthetised dogs. *Br. J. Pharmac.*, **47**, 196–205.

LAUBIE, M. (1975). Pharmacological evidence for interactions of cholinergic and noradrenergic mechanism in central cardiovascular control. *Proceedings of the Sixth Int. Congr. of Pharmac. (Helsinki), Drug Therapy*, **4**, 79–85.

NIJKAMP, F.P., EZER, J. & DE JONG, W. (1975). Central inhibitory effects of $\alpha$-methyldopa on blood pressure, heart rate and body temperature of renal hypertensive rats. *Eur. J. Pharmac.*, **31**, 243–249.

NIJKAMP, F.P. & DE JONG, W. (1975). $\alpha$-Methylnoradrenaline induced hypotension and bradycardia after administration into the area of the nucleus tractus solitarii. *Eur. J. Pharmac.*, **32**, 361–364.

PALKOVITZ, M. & JACOBOWITZ, D.M. (1974). Topographic atlas of catecholamine and acetylcholinesterase-containing neurons in the rat brain. II. Hindbrain (Mesencephalon, rhombencephalon). *J. comp. Neur.*, **157**, 29–42.

SATCHELL, D.G., FREEMAN, S.E. & HOPKINS, S.V. (1971). Effects of $\alpha$-methylnoradrenaline on cardiac metabolism. *Biochem. Pharmac.*, **20**, 1691–1694.

SCHMITT, H. (1975). On some unexplained effect of clonidine, In *Recent Advances in Hypertension II*, ed. Milliez, P. & Safar, M. pp. 63–74, Monaco: Boehringer and Ingelheim.

SCHMITT, H., SCHMITT, H., FENARD, S. & LAUBIE, M. (1973). Evidence for a sympathomimetic component inhibiting the sympathetic centres: Nature of the receptor. In *Symposium on Pharmacological Agents and Biogenic Amines in the Central Nervous System*. ed. Knoll, J. & Magyar, K. pp. 177–194, Budapest, Akademiai Kiado.

TODA, N., MATSUDA, Y. & SHIMAMOTO, K. (1969). Cardiovascular effects of sympathomimetic amines injected into the cerebral ventricles of rabbits *Int. J. Neuropharmac.*, **8**, 451–461.

VAN ZWIETEN, P.A. (1975). Antihypertensive drugs with a central action. *Progr. Pharmac.*, **1**, 1–63.

*(Received April 28, 1976.*  
*Revised June 22, 1976)*

*Br. J. Pharmac.* (1976), **58**, 599–603

# FURTHER EVIDENCE THAT PROSTAGLANDINS INHIBIT THE RELEASE OF NORADRENALINE FROM ADRENERGIC NERVE TERMINALS BY RESTRICTION OF AVAILABILITY OF CALCIUM

P. HEDQVIST
Department of Physiology, Karolinska Institutet, S-10401 Stockholm 60, Sweden

**1** Guinea-pig vasa deferentia were continuously superfused after labelling the transmitter stores with [$^3$H]-(–)-noradrenaline. Release of [$^3$H]-(–)-noradrenaline was induced by transmural nerve stimulation.

**2** Prostaglandin $E_2$ (14 nM) drastically reduced the release of [$^3$H]-(–)-noradrenaline, while tetraethylammonium (2 mM), rubidium (6 mM), phenoxybenzamine (3 µM) each in the presence or absence of Uptake $_1$ or $_2$ blockade, and prolonged pulse duration (from 0.5 to 2.0 ms) all significantly increased the release of [$^3$H]-(–)-noradrenaline per nerve impulse.

**3** The inhibitory effect of prostaglandin $E_2$ on evoked release of [$^3$H]-(–)-noradrenaline was significantly reduced by tetraethylammonium, rubidium and prolonged pulse duration, whilst it was actually enhanced by phenoxybenzamine. This indicates that increased release of noradrenaline per nerve impulse does not *per se* counteract the inhibitory effect of prostaglandin $E_2$.

**4** It is concluded that tetraethylammonium, rubidium and prolonged pulse duration counteracted the inhibitory effect of prostaglandin $E_2$ on [$^3$H]-(–)-noradrenaline release by promoting calcium influx during the nerve action potential. The results are consistent with, and add more weight to the view that prostaglandins inhibit the release of noradrenaline by restriction of calcium availability.

## Introduction

Ample experimental evidence indicates that prostaglandins of the E series may control adrenergic neuroeffector transmission by inhibiting noradrenaline (NA) release evoked by nerve action potentials. Such an action has been regularly observed in a large number of isolated tissues from different animal species and from man, and several *in vivo* studies point to the same mechanism of action (for references see, Hedqvist, 1970, 1976a; Wennmalm, 1971).

NA release by nerve action potentials is critically dependent upon calcium ions, and it is believed that depolarization of the nerve terminal causes an inward movement of calcium which in turn promotes focal extrusion of NA into the junctional cleft (*cf.* Simpson, 1968; Hubbard, 1970). Apparently calcium interacts with the inhibitory effect of prostaglandin E. Thus, increasing the calcium concentration in the perfusion medium to the cat spleen counteracts the inhibitory action of prostaglandin E on NA release and restores normal output (Hedqvist, 1970). The same interaction between calcium ions and prostaglandin E is also seen in the guinea-pig vas deferens, and in fact the inhibitory effect of prostaglandin E on NA release is progressively increased when the ambient calcium concentration is reduced (Hedqvist, 1973, 1974a; Stjärne, 1973a, b). Notably, NA release by tyramine, which is a calcium-independent process, is not affected by prostaglandin E (Hedqvist, 1970). In view of the decisive role of calcium in the process of stimulus secretion coupling these observations suggest that prostaglandin E inhibits NA release actually by restriction of calcium availability.

The present investigation was undertaken to probe further into the mechanism of action of prostaglandin $E_2$ on adrenergic neurotransmission. Experiments were carried out to determine the effect of increased pulse duration and of administration of tetraethylammonium (TEA) and rubidium ions on the inhibitory action of prostaglandin $E_2$ on NA release evoked by nerve stimulation in the guinea-pig vas deferens. The results show that any one of these treatments, considered to increase calcium influx during the nerve action potential, significantly reduces the inhibitory effect of prostaglandin $E_2$ on NA release. A preliminary account of part of the results has been published (Hedqvist, 1976b).

## Methods

Guinea-pigs, weighing 500–700 g, were killed by a blow on the head and the vasa deferentia were isolated

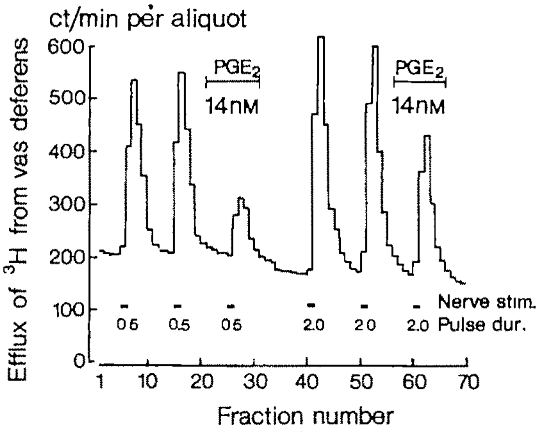


**Figure 1** Continuously superfused guinea-pig vas deferens, loaded with [$^3$H]-(–)-noradrenaline (NA). Inhibitory effect of prostaglandin $E_2$ (PGE$_2$) on release of tracer by transmural nerve stimulation (5 Hz, 450 pulses) at pulse durations of 0.5 ms or 2.0 ms. Fraction numbers = time in minutes.

and carefully dissected free from adjacent tissue. The isolated preparation was incubated for 1 h in Tyrode solution containing 10 µCi/ml of [$^3$H]-(–)-NA (sp. act. 5.4 Ci/mmol, N.E.N.) and was then thoroughly rinsed and continuously superfused in a 2 ml organ bath with NA-free Tyrode at a rate of 1 ml/minute. The composition of the solution was (mM): NaCl 136.7, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.4, glucose 5.5 and ascorbic acid 0.1. The solution was kept at 37°C and bubbled with 5% $CO_2$ in $O_2$. The preparation was electrically stimulated by means of platinum electrodes in the wall of the bath and a Grass S4 stimulator delivering trains of biphasic pulses (5 Hz, 0.5–2.0 ms duration, 450 pulses, supramaximal voltage) at 10 min intervals. The superfusate was divided into 1 min samples and the radioactivity was determined by counting 0.5–1.0 ml aliquots in a Packard liquid scintillation spectrometer using Instagel (Packard Instr. Co.) as counting medium. Quenching was monitored by internal standards.

**Table 1** Superfused guinea-pig vasa deferentia, loaded with [$^3$H]-(–)-noradrenaline (NA) and subjected to transmural nerve stimulation (5 Hz, 450 pulses). Percentage increase in evoked release of [$^3$H]-(–)-NA by raising the pulse duration from 0.5 to 2.0 ms, and by administration of tetraethylammonium (2 mM) or rubidium (6 mM) (with pulse duration kept at 1 ms).

| *% increase in evoked [$^3$H]-(–)-Na release* | | |
|---|---|---|
| *Pulse duration* | *Tetraethylammonium* | *Rubidium* |
| 101 ± 23 (8)<br>$P<0.01$ | 165 ± 12 (5)<br>$P<0.001$ | 77 ± 13 (5)<br>$P<0.01$ |

Mean values ± s.e. of 5 to 8 experiments.

The following drugs were used: desipramine hydrochloride, indomethacin, (±)-normetanephrine hydrochloride, phenoxybenzamine hydrochloride, prostaglandin $E_2$, rubidium chloride and tetraethylammonium chloride.

Experimental data were expressed as means ± s.e. Significance was calculated according to Student's *t*-test for paired and unpaired data.

## Results

The superfused guinea-pig vas deferens, in which the NA stores were prelabelled with [$^3$H]-(–)-NA, was transmurally stimulated (5 Hz, 0.5–2.0 ms, 450 pulses supramaximal voltage) at 10 min intervals. This type of stimulation, which specifically activates nerve fibres, causes an increased overflow of tracer, the bulk of which consists of intact [$^3$H]-(–)-NA (Hedqvist, 1974b).

With other stimulation parameters kept constant, the overflow of [$^3$H]-(–)-NA was found to vary with the pulse duration. Thus, increasing the pulse duration from 0.5 to 2.0 ms increased the overflow of [$^3$H]-(–)-NA by 101% (Table 1). Prostaglandin $E_2$ (14 nM) in the superfusing solution, markedly and reversibly inhibited the overflow of [$^3$H]-(–)-NA in response to transmural nerve stimulation at a pulse duration of 0.5 ms (Figure 1). The inhibition by prostaglandin $E_2$ remained essentially the same when this procedure was repeated several times. On the other hand, increasing the pulse duration to 2 ms significantly reduced the inhibitory action of prostaglandin $E_2$ on evoked [$^3$H]-(–)-NA overflow (Figure 1 and Table 2).

In agreement with observations on the cat spleen (Thoenen, Haefely & Staehelin, 1967; Kirpekar, Prat, Puig & Wakade, 1972), TEA (2 mM) and rubidium (6 mM) significantly increased the overflow of [$^3$H]-(–)-NA induced by transmural nerve stimulation (5 Hz, 1 ms, 450 pulses) (Table 1), without affecting the spontaneous efflux of tracer. The enhancing effect of TEA and rubidium remained after combined treatment with desipramine (0.5 µM) and normetanephrine (1 µM), given in order to block NA uptake mechanisms. TEA and rubidium were also tested with respect to their capacity to interfere with the effects of prostaglandin $E_2$ on NA release. Both compounds were found to reduce significantly the inhibitory action of prostaglandin $E_2$ on [$^3$H]-(–)-NA overflow induced by transmural nerve stimulation (Table 2).

Figure 2 (typical of 3 experiments) illustrates the inhibitory effect of prostaglandin $E_2$ on overflow of [$^3$H]-(–)-NA induced by transmural nerve stimulation, before and after administration of the α-adrenoceptor blocking agent, phenoxybenzamine (PB). PB (3 µM)

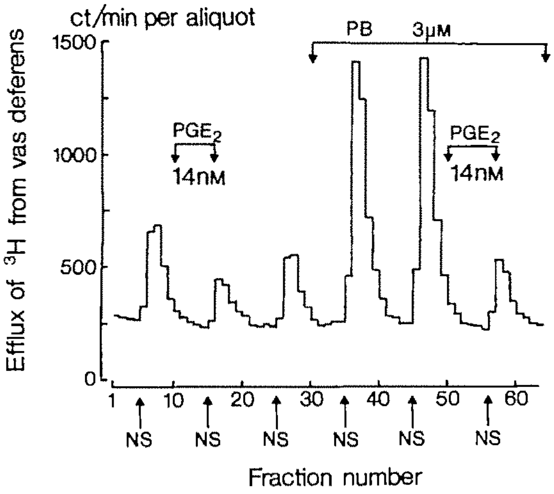


**Figure 2** Continuously superfused guinea-pig vas deferens, loaded with [$^3$H]-(–)-noradrenaline (NA). Effect of prostaglandin $E_2$ ($PGE_2$) on release of tracer by transmural nerve stimulation (NS) (5 Hz, 1 ms, 450 pulses) before and after phenoxybenzamine (PB) treatment. Fraction numbers = time in minutes.

markedly increased the overflow of [$^3$H]-(–)-NA induced by transmural nerve stimulation (5 Hz, 1 ms, 450 pulses), but it did not reduce the inhibitory effect of prostaglandin $E_2$. In fact, after PB the preparation proved even more sensitive to prostaglandin $E_2$, the calculated inhibition being 48% and 73%, respectively before and after PB. After treatment with desipramine (0.5 μM) and normetanephrine (1 μM), PB increased the stimulated overflow of [$^3$H]-(–)-NA by 209 ± 33% (mean ± s.e., $n = 4$), indicating that the enhancement was not due to blockade of NA uptake mechanisms. In an additional 4 experiments, in which indomethacin (6 μM) was given in order to block local prostaglandin formation, prostaglandin $E_2$ remained more effective (15 ± 4%, mean ± s.e., $P < 0.05$) in inhibiting evoked [$^3$H]-(–)-NA overflow after PB treatment.

## Discussion

The present study showed that prolongation of the pulse duration or administration of TEA or rubidium ions significantly depressed the inhibitory effect of prostaglandin $E_2$ on [$^3$H]-(–)-NA release induced by transmural nerve stimulation in the guinea-pig vas deferens. In addition, these treatments significantly enhanced the evoked overflow of [$^3$H]-(–)-NA, as previously shown for TEA and rubidium in the cat spleen (Thoenen *et al.*, 1967; Kirpekar *et al.*, 1972). It appears likely that this enhancement actually means increased NA release per nerve impulse rather than altered disposition of released transmitter. Thus, the effect of TEA and rubidium remained after blockade of NA uptake mechanisms. Moreover, it has been shown that the tracer overflowing from the vas deferens in response to transmural nerve stimulation in the absence of drug treatment consists almost wholly of intact [$^3$H]-(–)-NA (Hedqvist, 1974b).

Considerable circumstantial evidence suggests that E prostaglandins inhibit adrenergic neurotransmission by reducing the influx of calcium necessary for the ultimate extrusion of NA into the junctional cleft. Thus, the inhibitory action of prostaglandin E varies inversely with the ambient calcium concentration (Hedqvist, 1970, 1973, 1974a; Stjärne, 1973a, b). On the other hand, NA release by tyramine, which is a calcium-independent process, is unaffected by prostaglandin E (Hedqvist, 1970). It is also worth noticing that the inhibitory action of prostaglandin E on NA release per nerve impulse is diminished by increasing the pulse frequency (Hedqvist, 1973; Stjärne, 1973c; Frame & Hedqvist, 1975), and shortening the pulse interval is considered to leave

**Table 2** Superfused guinea-pig vasa deferentia, loaded with [$^3$H]-(–)-noradrenaline (NA) and subjected to transmural nerve stimulation (5 Hz, 1 ms, 450 pulses). Effect of increased pulse duration, administration of tetraethylammonium (TEA) (2 mM), or rubidium (6 mM) on inhibitory action of prostaglandin $E_2$ ($PGE_2$, 14 nM) on evoked [$^3$H]-(–)-NA release.

| *No. of expts* | *Drug treatment* | *Pulse duration (ms)* | *Inhibition by $PGE_2$ (%)* | *Paired difference (%)* |
|---|---|---|---|---|
| 8 | 0<br>0 | 0.5<br>2.0 | 61.2 ± 7.2<br>38.0 ± 6.6 | 23.2 ± 4.7 $P < 0.01$ |
| 5 | 0<br>Rubidium | 1.0<br>1.0 | 56.9 ± 5.0<br>41.2 ± 5.7 | 15.7 ± 2.8 $P < 0.01$ |
| 5 | 0<br>TEA | 1.0<br>1.0 | 52.4 ± 4.2<br>19.6 ± 2.4 | 32.8 ± 6.3 $P < 0.01$ |

Mean values ± s.e. of 5 to 8 experiments.

more residual calcium at the active releasing sites in the axon (Katz & Miledi, 1968). In addition, prostaglandin E has been reported to inhibit the uptake of calcium into membranes, and also to facilitate its efflux from membranes (Ramwell & Shaw, 1970; Alm, Efendic & Löw, 1970; Kirtland & Baum, 1972; Carafoli & Crovetti, 1973).

Increased release of transmitter by prolonging the nerve action potential has been considered due to more calcium entering the axon (Katz & Miledi, 1967a, b; Hubbard, 1970). Evidently, TEA and rubidium ions also prolong the nerve action potential (Koketsu, 1958; Baker, Hodgkin & Shaw, 1962), and their enhancing effect on evoked NA release may likewise be explained in terms of an action on calcium influx.

Kirpekar *et al.* (1972) have, as an alternative, explained enhanced NA release by TEA and rubidium as a result of their capacity to inactivate potassium channels (*cf.* Katz & Miledi, 1969), assuming that the outward movement of potassium during the nerve action potential controls the release of NA by modulating the calcium entry into the axon. However, there seems to be little independent evidence to suggest that TEA and rubidium interact with prostaglandin $E_2$ at the level of the potassium channels. In the experiments of Kirpekar *et al.* (1972), TEA, 1 and 10 mM, were found to cause the same and maximal enhancement of NA release. Supposedly, this means complete inactivation of the potassium channels, as is the case in cholinergic nerves (Hille, 1967; Katz & Miledi, 1969). Yet, in the present experiments prostaglandin $E_2$ was able to cause a marked inhibition of evoked NA release in the presence of 2 mM TEA. It has also been shown that prostaglandin $E_2$ markedly inhibits the release of NA evoked by high potassium (Stjärne, 1973d). Therefore, the effect of TEA and rubidium, like that of increased pulse duration, on prostaglandin $E_2$-induced inhibition of NA release seems to be best explained in terms of an action on the calcium gates allowing more calcium to enter the axon.

Admittedly, increased pulse duration or administration of TEA or rubidium could counteract the inhibitory action of prostaglandin $E_2$ secondarily to the increased release of NA per nerve impulse. This possibility was ruled out by the finding that PB, which increased the overflow of NA as efficiently as any of the above mentioned treatments, failed to counteract the inhibitory effect of prostaglandin $E_2$ and rather increased it (cf. Hedqvist, 1974b). It may be noted that the enhancing effect of PB on evoked NA release remains after blockade of NA uptake mechanisms, and that it in all probability represents increased NA release per nerve impulse by blockade of prejunctional $\alpha$-adrenoceptors (Farnebo & Malmfors, 1971; Enero, Langer, Rothlin & Stefano, 1972; Starke, 1972; Hedqvist, 1973, Stjärne, 1973e).

The increased sensitivity to the inhibitory effect of prostaglandin $E_2$ after $\alpha$-adrenoceptor blockade invites a comment. Stjärne (1973f) has proposed that prejunctional $\alpha$-adrenoceptors control NA release by restriction of availability of external calcium for the secretory mechanism. If this is the case, inhibition of the $\alpha$-adrenoceptors by PB would increase the availability of calcium, which, being reasonably equivalent to raising the ambient calcium concentration, would be expected to counteract the inhibitory action of prostaglandin $E_2$ rather than causing the observed opposite effect. Another aspect of this phenomenon is that prostaglandin synthesis inhibitors have been shown to increase the inhibitory effect of prostaglandin $E_2$ (Hedqvist, 1974b; Frame & Hedqvist, 1975), and PB can in fact inhibit prostaglandin release induced by sympathetic nerve stimulation (Davies, Horton & Withrington, 1967). However, such an action can not explain the increased sensitivity to prostaglandin $E_2$ in the presence of PB, since, as observed in the present study, it remained after blockade of local prostaglandin formation by indomethacin.

Irrespective of whether the ambient calcium concentration is augmented or the pulse frequency is increased, or, as in the present study, pulse duration is prolonged, or TEA or rubidium ions are administered, there is a decrease in the inhibitory action of prostaglandin $E_2$ on evoked NA release. One common denominator of all these different treatments seems to be increased availability of calcium at the active NA releasing sites in the axon, and, therefore, it appears highly likely that prostaglandin $E_2$ interferes with the NA release process actually by inhibiting calcium from reaching these active sites.

This study was supported by grants from the Swedish Medical Research Council, project no. 04X-4342.

## References

ALM, B., EFENDIC, S. & LÖW, H. (1970). Effect of lipolytic and antilipolytic agents on the uptake of $^{47}$calcium into rat adipose tissue *in vitro*. *Hormone metabol. Res.*, **2**, 142–146.

BAKER, P.F., HODGKIN, A.L. & SHAW, T.I. (1962). The effect of changes in internal ionic concentrations on the electrical properties of perfused giant axons. *J. Physiol., Lond.*, **164**, 335–374.

CARAFOLI, E. & CROVETTI, F. (1973). Interactions between prostaglandin $E_1$ and calcium at the level of the mitochondrial membrane. *Arch. Biochem.*, **154**, 40–46.

DAVIES, B.N., HORTON, E.W. & WITHRINGTON, P.G. (1967). The occurrence of prostaglandin $E_2$ in splenic venous blood of the dog following splenic nerve stimulation. *J. Physiol., Lond.*, **188**, 38–39P.

ENERO, M.A., LANGER, S.Z., ROTHLIN, R.P. & STEFANO, F.J.E. (1972). Role of the $\alpha$-adrenoceptor in regulating noradrenaline overflow by nerve stimulation. *Br. J. Pharmac.*, **44**, 672–688.

FARNEBO, L.O. & MALMFORS, T. (1971). $^3$H-noradrenaline release and mechanical response in the field stimulated mouse vas deferens. *Acta physiol. scand.*, Suppl. **371**, 1–18.

FRAME, M.H. & HEDQVIST, P. (1975). Evidence for prostaglandin mediated prejunctional control of renal sympathetic transmitter release and vascular tone. *Br. J. Pharmac.*, **54**, 189–196.

HEDQVIST, P. (1970). Studies on the effect of prostaglandins $E_1$ and $E_2$ on the sympathetic neuromuscular transmission in some animal tissues. *Acta physiol. scand.*, **79**, Suppl. 345, 1–40.

HEDQVIST, P. (1973). Aspects of prostaglandin and $\alpha$-receptor mediated control of transmitter release from adrenergic nerves. In *Frontiers in Catecholamine Research*, ed. Usdin, E. & Snyder, S., pp. 583–587. Oxford and New York: Pergamon Press.

HEDQVIST, P. (1974a). Interaction between prostaglandins and calcium ions on noradrenaline release from the stimulated guinea pig vas deferens. *Acta physiol. scand.*, **90**, 153–157.

HEDQVIST, P. (1974b). Prostaglandin action on noradrenaline release and mechanical responses in the stimulated guinea pig vas deferens. *Acta physiol. scand.*, **90**, 86–93.

HEDQVIST, P. (1976a). Effects of prostaglandins on autonomic neurotransmission. In *Prostaglandins —Physiological, Pharmacological and Pathological Aspects*, ed. Karim, S.M.M., pp. 37–61. Lancaster: MTP Co.

HEDQVIST, P. (1976b). Prostaglandin action on transmitter release at adrenergic neuroeffector junctions. In *Advances in Prostaglandin and Thromboxane Research*, Vol. 1, ed. Samuelsson, B. & Paoletti, R., pp. 357–363. New York: Raven Press.

HILLE, B. (1967). The selective inhibition of delayed potassium currents in nerve by tetraethylammonium. *J. gen. Physiol.*, **50**, 1287–1302.

HUBBARD, J.I. (1970). Mechanism of transmitter release. *Progr. Biophys. mol. Biol.*, **21**, 33–124.

KATZ, B. & MILEDI, R. (1967a). The release of acetylcholine from nerve endings by graded electric pulses. *Proc. Roy. Soc. B.*, **167**, 23–28.

KATZ, B. & MILEDI, R. (1967b). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol., Lond.*, **192**, 407–436.

KATZ, B. & MILEDI, R. (1968). The role of calcium in neuromuscular facilitation. *J. Physiol., Lond.*, **195**, 481–492.

KATZ, B. & MILEDI, R. (1969). Tetrodotoxin resistant electric activity in presynaptic terminals. *J. Physiol., Lond.*, **203**, 459–487.

KIRPEKAR, S.M., PRAT, J.C., PUIG, M. & WAKADE, A.R. (1972). Modification of the evoked release of noradrenaline from the perfused cat spleen by various ions and agents. *J. Physiol., Lond.*, **221**, 601–615.

KIRTLAND, S.J. & BAUM, H. (1972). Prostaglandin $E_1$ may act as a 'calcium ionophore'. *Nature New Biol.*, **236**, 47–49.

KOKETSU, K. (1958). Action of tetraethylammonium chloride on neuromuscular transmission in frogs. *Amer. J. Physiol.*, **193**, 213–215.

RAMWELL, P.W. & SHAW, J.E. (1970). Biological significance of the prostaglandins. *Rec. Progr. Hormone Res.*, **26**, 139–173.

SIMPSON, L.L. (1968). The role of calcium in neurohumoral and neurohormonal extrusion processes. *J. Pharm. Pharmac.*, **20**, 889–910.

STARKE, K. (1972). Influence of extracellular noradrenaline on the stimulation-evoked secretion of noradrenaline from sympathetic nerves: evidence for an $\alpha$-receptor-mediated feed-back inhibition of noradrenaline release. *Naunyn-Schmiedebergs Arch. Pharmac.*, **275**, 11–23.

STJÄRNE, L. (1973a). Inhibitory effect of prostaglandin $E_2$ on noradrenaline secretion from sympathetic nerves as a function of external calcium. *Prostaglandins*, **3**, 105–109.

STJÄRNE, L. (1973b). Kinetics of secretion of sympathetic neurotransmitter as a function of external calcium: mechanism of inhibitory effect of prostaglandin E. *Acta physiol. scand.*, **87**, 428–430.

STJÄRNE, L. (1973c). Frequency dependence of dual negative feedback control of secretion of sympathetic neurotransmitter in guinea-pig vas deferens. *Br. J. Pharmac.*, **49**, 358–360.

STJÄRNE, L. (1973d). Comparison of secretion of sympathetic neurotransmitter induced by nerve stimulation with that evoked by high potassium, as triggers of dual $\alpha$-adrenoceptor mediated negative feed-back control of noradrenaline secretion. *Prostaglandins*, **3**, 421–426.

STJÄRNE, L. (1973e). $\alpha$-adrenoceptor mediated feed-back control of sympathetic neurotransmitter secretion in guinea-pig vas deferens. *Nature New Biol.*, **241**, 190–191.

STJÄRNE, L. (1973f). Michaelis-Menten kinetics of secretion of sympathetic neurotransmitter as a function of external calcium: Effect of graded $\alpha$-adrenoceptor blockade. *Naunyn-Schmiedebergs Arch. Pharmac.*, **278**, 323–327.

THOENEN, H., HAEFELY, W. & STAEHELIN, H. (1967). Potentiation by tetraethylammonium of the response of the cat spleen to postganglionic sympathetic nerve stimulation. *J. Pharmac. exp. Ther.*, **157**, 532–540.

WENNMALM, Å. (1971). Studies on mechanisms controlling the secretion of neurotransmitters in the rabbit heart. *Acta physiol. scand.*, Suppl. **365**, 1–36.

*(Received May 3, 1976.*
*Revised June 3, 1976)*

*Br. J. Pharmac.* (1976), **58**, 605–612

# THE EFFECT OF DIBUTYRYL CYCLIC ADENOSINE-3′,5′-MONOPHOSPHATE ON PROTEIN SECRETION FROM THE RAT EXOCRINE PANCREAS *in vitro*

D. FAST[1] & A. TENENHOUSE
Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada

1 The mechanism by which dibutyryl cyclic adenosine-3′-5′-monophosphate (dibutyryl cyclic AMP) potentiates the secretory effect of carbachol in rat exocrine pancreas was investigated.

2 Dibutyryl cyclic AMP potentiated the secretory effect of carbachol only at carbachol concentrations $\overline{>} 10^{-7}$ mol/l; was independent of carbachol at concentrations $> 10^{-7}$ mol/l and was inversely proportional to extracellular [$Ca^{2+}$].

3 Carbachol increased and dibutyryl cyclic AMP reduced the rate of $^{45}Ca$ efflux from the tissue.

4 A-23187 stimulated $^{3}H$-protein release in the presence of $Ca^{2+}$ and this effect was potentiated by dibutyryl cyclic AMP; the degree of potentiation was inversely proportional to extracellular [$Ca^{2+}$]. At $10^{-3}$ mol/l [$Ca^{2+}$] the potentiation occurred only at ionophore concentrations $\overline{<} 10^{-6}$ mol/litre.

5 These results support the hypothesis that dibutyryl cyclic AMP potentiates the effect of secretagogues in rat exocrine pancreas by maintaining an elevated intracellular calcium concentraion. It does so by inhibiting $Ca^{2+}$ efflux. The results also suggest that the limiting factor in carbachol-stimulated secretion, at all concentrations of carbachol, is intracellular [$Ca^{2+}$].

## Introduction

The precise mechanism of hormone-stimulated enzyme secretion from the exocrine pancreas is not yet known but there is little doubt that a critically important element in this process is calcium (Hokin, 1966; Robberecht & Christophe, 1971; Heisler, Fast & Tenenhouse, 1972; Case & Scratcherd, 1972). The most convincing recent evidence is the demonstration by Eimerl, Savion, Heichel & Selinger (1974) and Williams & Lee (1974) that ionophore A-23187, in the presence of calcium, stimulates amylase secretion from the rat exocrine pancreas *in vitro*. The involvement of cyclic nucleotides in this process is still controversial, however the bulk of evidence suggests that cyclic adenosine-3′,5′-monophosphate (cyclic AMP) is not involved in either acetylcholine or pancreozymin-stimulated enzyme secretion (Benz, Eckstein, Matthews & Williams, 1972; Case & Scratcherd, 1972; Heisler *et al.*, 1972). It has been suggested that cyclic GMP mediates the effects of acetylcholine in some tissues (Goldberg, O'Dea & Haddox, 1973) including the exocrine pancreas (Robberecht, Deschodt-Lanckman, De Neef, Borgeat & Christophe, 1974) but the evidence that this nucleotide plays a role in secretion from this tissue is preliminary.

Heisler *et al.* (1972) reported that dibutyryl cyclic AMP was itself a poor secretagogue in the rat exocrine pancreas but that it potentiated the secretory effect of carbachol. This effect was seen at carbachol concentrations that were optimum for secretion and was dependent on extracellular calcium. To explain these observations we proposed that activation of secretion requires an elevation of cytoplasmic ionized calcium. This has been suggested by many investigators for a number of secretory systems (Douglas, 1968; Rasmussen & Tenenhouse, 1968; Rasmussen, 1970; Rasmussen, Goodman & Tenenhouse, 1972) and is now widely accepted. In the case of cholinomimetic secretagogues in the exocrine pancreas the rate limiting factor at optimum concentration of secretagogue is the concentration of cytoplasmic ionized calcium. Dibutyryl cyclic AMP potentiates the activity of these secretagogues by conserving cytoplasmic $Ca^{2+}$, probably by inhibiting those processes which remove $Ca^{2+}$ from the cytoplasm. The experiments described in this paper were designed to test this hypothesis.

## Methods

Female Wistar rats weighing 225 to 250 g were anaesthetized with ether and killed by decapitation.

---

[1] Present address: Pathophysiologische Institut, Universität Bern, Bern, Switzerland.

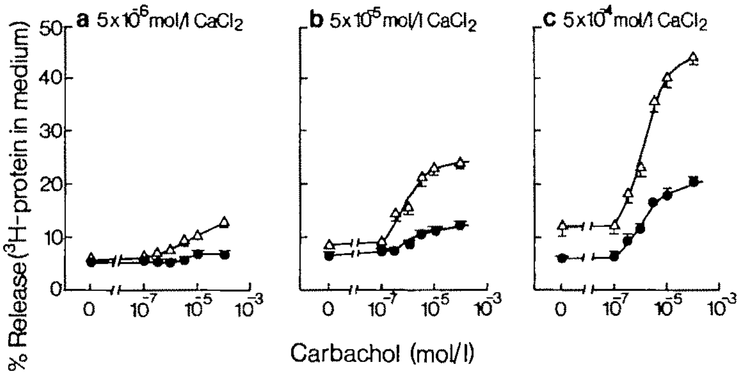


**Figure 1** The effect of varying the concentration of carbachol on tritiated protein release from rat pancreas pieces in the presence (△) or absence (●) of $10^{-3}$ mol/l dibutyryl cyclic AMP. EGTA ($5 \times 10^{-5}$ mol/l) plus (a) $5 \times 10^{-6}$ mol/l $Ca^{2+}$, (b) $5 \times 10^{-5}$ mol/l $Ca^{2+}$ or (c) $5 \times 10^{-4}$ mol/l $Ca^{2+}$ was present in the Krebs-Ringer bicarbonate buffer as described in the text. Each point is the mean of at least five experimental values; standard errors are indicated by the vertical lines.

The pancreases were quickly removed and placed in ice-cold Krebs-Ringer bicarbonate buffer of the following composition (mM): NaCl 118.5, KCl 4.75, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, and $NaHCO_3$ 25.0. Calcium ($CaCl_2$) and EGTA (ethylene glycol bis (β-aminoethyl ether)-N, N′-tetraacetic acid) were added as indicated in the results section. The buffer also contained 10 mM glucose and L-amino acids (with the exception of leucine) in concentrations recommended by Eagle (1959).

The tissue was trimmed free of adherent fat and connective tissue in chilled (ice-cold) Krebs-Ringer bicarbonate buffer and minced with scissors into 5 to 15 mg pieces. The proteins of pancreatic zymogen granules were labelled with [$^3$H]-leucine as described by Jamieson & Palade (1967) and the release of radioactive proteins from the pancreas measured as described previously (Heisler *et al.*, 1972).

For investigations of $^{45}$Ca efflux, the pancreatic pieces were first incubated for 90 min in Krebs-Ringer bicarbonate buffer containing 0.5 μCi/ml $^{45}$Ca and sufficient non-radioactive $CaCl_2$ to make the final calcium concentration $10^{-4}$ mol/litre. The tissue and medium were filtered through cheesecloth and the tissue washed with Krebs-Ringer bicarbonate buffer. The pancreas pieces were divided into 180 to 200 mg portions; these were transferred to flasks of buffered medium containing $10^{-4}$ mol/l non-radioactive $Ca^{2+}$, $7.5 \times 10^{-5}$ mol/l EGTA, and the stimulating agent(s) to be tested in a final volume of 3 ml. Aliquots (10 μl) of medium were removed at various times and the radioactivity determined in a liquid scintillation counter. At the end of the incubation the tissue was solubilized in NCS and an aliquot counted to determine the tissue content of $^{45}$Ca. Results are expressed as the percentage of total tissue $^{45}$Ca released into the medium.

Each experiment was done at least three times. Results are given for typical experiments or as the mean of several (at least five) experiments plus or minus the standard error. The significance of differences was determined using Student's test. The ionophore A-23187 was a gift of Dr R.L. Hamill, Lilly Research Laboratories, Lilly & Co., Indianapolis, Indiana, U.S.A.

## Results

The effect of varying extracellular calcium concentration on tritiated protein secretion from the pancreas was studied at various carbachol concentrations, using carbachol alone or in combination with dibutyryl cyclic AMP $10^{-3}$ mol/litre. Based on the results of previous experiments (Heisler *et al.*, 1972) three concentrations of calcium were chosen for these experiments: $5 \times 10^{-6}$ mol/l $Ca^{2+}$, at which concentration carbachol alone did not significantly stimulate secretion; $5 \times 10^{-5}$ mol/l, the lowest $Ca^{2+}$ concentration at which carbachol-dependent secretion could be detected; $5 \times 10^{-4}$ mol/l, at which concentration $^3$H-protein release caused by carbachol was near maximum. The results of these experiments, shown in Figure 1, give the per cent of total $^3$H-protein released into the medium by the tissue as a function of the carbachol concentration.

At concentrations of carbachol less than $10^{-7}$ mol/l there was no stimulation of radioactive protein release at any of the calcium concentrations tested. In combination with dibutyryl cyclic AMP $10^{-3}$ mol/l, carbachol $10^{-7}$ mol/l or less did not increase $^3$H-protein release above that caused by the cyclic nucleotide alone; i.e., there was no effect of carbachol plus dibutyryl cyclic AMP until the carbachol con-

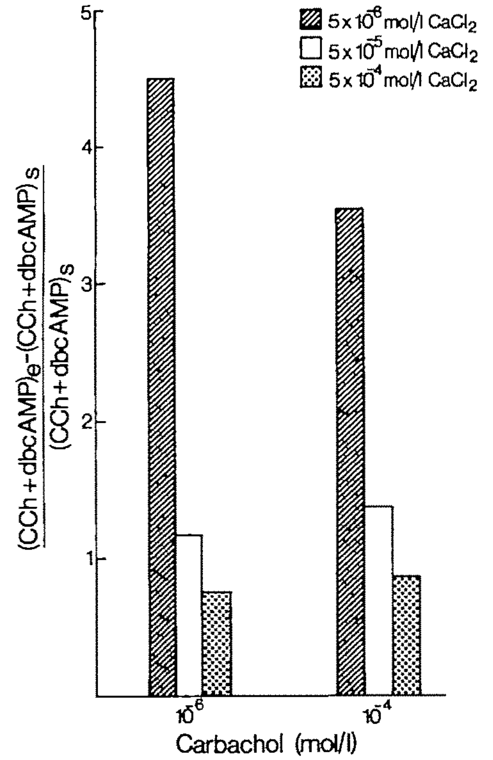


**Figure 2** The degree of potentiation of carbachol (CCh)-stimulated $^3$H-protein release from rat pancreas by dibutyryl cyclic AMP (dbc AMP) at three [Ca$^{2+}$], 5 x 10$^{-6}$, 5 x 10$^{-5}$ and 5 x 10$^{-4}$ mol/litre.

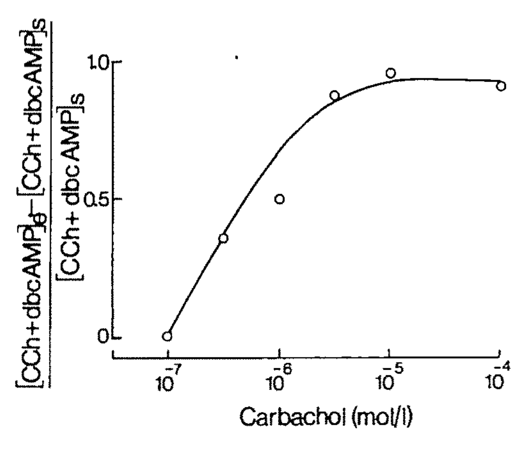


**Figure 3** The degree of potentiation of carbachol (CCh)-stimulated $^3$H-protein release from rat exocrine pancreas by dibutyryl cyclic AMP (dbc AMP, 10$^{-3}$ mol/l) as a function of carbachol concentration, [Ca$^{2+}$], 10$^{-3}$ mol/litre.

centration was greater than 10$^{-7}$ mol/litre. Carbachol (5 x 10$^{-7}$ mol/l) stimulated $^3$H-protein release at 5 x 10$^{-5}$ mol/l Ca$^{2+}$ to 7.3% ($P<0.05$ compared to non-stimulated release) and at 5 x 10$^{-4}$ mol/l Ca$^{2+}$ to 9.6% ($P<0.01$). Tritiated protein release caused by any of the concentrations of carbachol used in the absence of dibutyryl cyclic AMP was not significantly different from non-stimulated release at 5 x 10$^{-6}$ mol/l Ca$^{2+}$. Also at 5 x 10$^{-7}$ mol/l carbachol and at all three concentrations of calcium tested, the stimulatory effect (% total $^3$H-protein minus % total $^3$H-protein released in control) of carbachol plus dibutyryl cyclic AMP was greater than the sum of the stimulatory effects of the individual agents. At 5 x 10$^{-5}$ mol/l Ca$^{2+}$, for example, the stimulatory effect of 5 x 10$^{-7}$ mol/l carbachol was 0.8%, that of 10$^{-3}$ mol/l dibutyryl cyclic AMP was 2.0% and the stimulatory effect of carbachol plus dibutyryl cyclic AMP was 8.0%.

Figure 2 illustrates the degree of potentiation of carbachol-dependent $^3$H-protein secretion caused by dibutyryl cyclic AMP 10$^{-3}$ mol/litre. This value was calculated from the results given in Figure 1 to determine whether the degree of potentiation was a function of the concentration of carbachol and/or calcium. The value $(\mathrm{CCh+dbcAMP})_e$, the experimental value for $^3$H-protein release caused by carbachol plus dibutyryl cyclic AMP, was the per cent of $^3$H-protein release caused by carbachol plus dibutyryl cyclic AMP minus $^3$H-protein release with no added stimulus, i.e. the stimulatory effect of carbachol plus dibutyryl cyclic AMP. The theoretical value for secretion caused by carbachol and dibutyryl cyclic AMP (the sum of their individual effect), $(\mathrm{CCh+dbcAMP})_s$, was calculated by adding the stimulatory effect of carbachol alone to that of dibutyryl cyclic AMP alone. The degree of potentiation was calculated from the formula:

$$(\mathrm{CCh + dbc\ AMP})_e - (\mathrm{CCh + dbc\ AMP})_s \,/(\mathrm{CCh + dbc\ AMP})_s$$

The degree of potentiation was calculated at 10$^{-6}$ and 10$^{-4}$ mol/l carbachol and at 5 x 10$^{-6}$, 5 x 10$^{-5}$ and 5 x 10$^{-4}$ mol/l calcium. At 5 x 10$^{-6}$ mol/l Ca$^{2+}$ the degree of potentiation was 4.5 and 3.5 for 10$^{-6}$ mol/l and 10$^{-4}$ mol/l carbachol respectively; at 5 x 10$^{-5}$ mol/l Ca$^{2+}$ it was 1.2 and 1.4 for these concentrations of carbachol; at 5 x 10$^{-4}$ mol/l Ca$^{2+}$ it was 0.5 and 0.9.

In Figure 3 the degree of potentiation, as a function of carbachol concentration is illustrated. In these experiments the extracellular Ca$^{2+}$ and the dibutyryl cyclic AMP concentrations were 10$^{-3}$ mol/l and the carbachol concentration varied between 10$^{-7}$ and 10$^{-4}$ mol/litre. As can be seen the degree of potentiation which is zero at 10$^{-7}$ mol/l carbachol, increased to 0.88 as the carbachol concentration

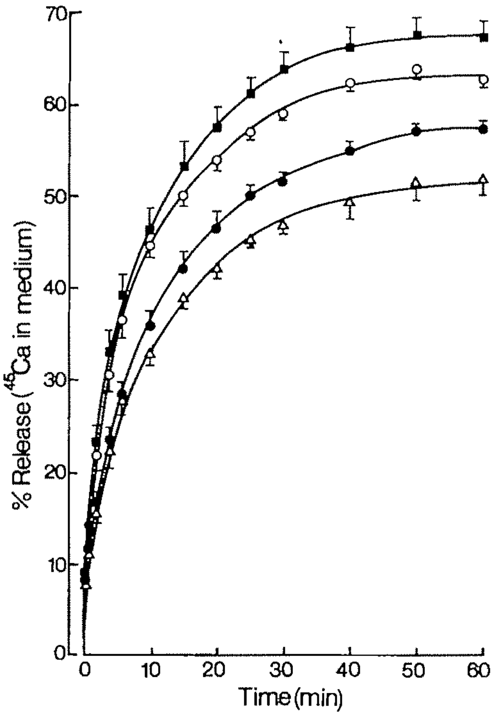


**Figure 4** The effect of $10^{-3}$ mol/l dibutyryl cyclic AMP (△), $10^{-5}$ mol/l carbachol (■) or the combination of carbachol plus dibutyryl cyclic AMP (○) on the rate of $^{45}$Ca efflux from rat pancreas pieces (control = ●). Each point is the mean of at least five separate experimental values; standard errors are indicated by the vertical lines. Details are given in the text.

increased to $5 \times 10^{-5}$ mol/litre. Further increase in carbachol concentration had no effect on the degree of potentiation.

The rates of uptake and release of $^{45}$Ca from pancreatic cells was measured to evaluate the role of calcium transport in protein secretion stimulated by carbachol or dibutyryl cyclic AMP. No difference in the rate of radioactive calcium uptake was observed when carbachol ($10^{-5}$ mol/l), dibutyryl cyclic AMP ($10^{-3}$ mol/l) or a combination of the two agents was added to the medium (unpublished observation).

Efflux of $^{45}$Ca from prelabelled tissue was measured as described in the Methods section, and the results of these experiments are shown in Figure 4.

Carbachol alone or a combination of carbachol plus dibutyryl cyclic AMP caused a significant increase in $^{45}$Ca efflux ($P < 0.05$ compared to control values) as early as 2 min after the addition of the stimulus. At 2 min $^{45}$Ca efflux caused by carbachol was 23.0% and efflux caused by carbachol plus dibutyryl cyclic AMP was 21.8%; control efflux was 16.8%. Dibutyryl cyclic AMP alone decreased the amount of $^{45}$Ca released into the medium. This effect of dibutyryl cyclic AMP became significant ($P < 0.05$) after 25 min of stimulation, when efflux caused by dibutyryl cyclic AMP was 45.4% and control efflux was 50.0%. Although radioactive calcium efflux caused by carbachol plus dibutyryl cyclic AMP was always less than that with carbachol alone, this difference was not statistically significant if each time point was regarded as a separate entity. However the probability that twelve points for $^{45}$Ca efflux caused by carbachol plus dibutyryl cyclic AMP would be less than thirteen points for $^{45}$Ca efflux with carbachol alone due entirely to chance, was less than 0.002 using Binomial Theorem. The combination of carbachol plus dibutyryl cyclic AMP, therefore, was having a significantly different effect from carbachol alone on the efflux of $^{45}$Ca. Radioactive calcium efflux reached a plateau after 40 min for both control and stimulated efflux. Efflux at 40 min was 55.1% of total $^{45}$Ca for the control, 49.5% for dibutyryl cyclic AMP, 66.0% for carbachol and 62.4% for carbachol plus dibutyryl cyclic AMP. Figure 5 shows that the effects of carbachol and carbachol plus dibutyryl cyclic AMP on $^{45}$Ca efflux are abolished by atropine ($10^{-6}$ mol/litre).

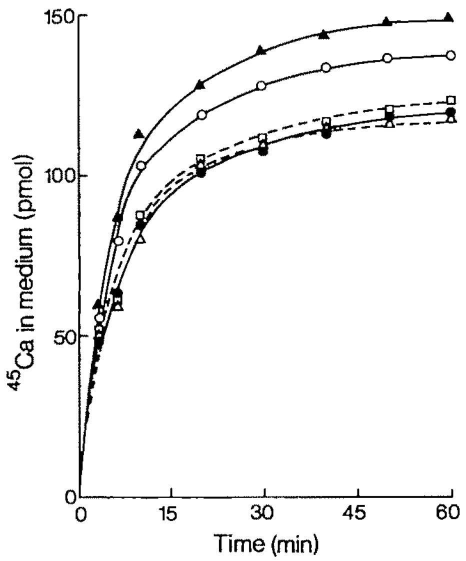


**Figure 5** The effect of atropine on carbachol, or carbachol plus dibutyryl cyclic AMP, stimulated $^{45}$Ca efflux from rat pancreas pieces. (●) Control; (▲) carbachol $10^{-5}$ mol/l; (○) carbachol + dibutyryl cyclic AMP $10^{-3}$ mol/l; (△) carbachol + atropine $10^{-6}$ mol/l; (□) carbachol + dibutyryl cyclic AMP + atropine.

The ionophore A-23187 is believed to insert itself into cell membranes and act as a freely mobile carrier to equilibrate the concentration of divalent cations,

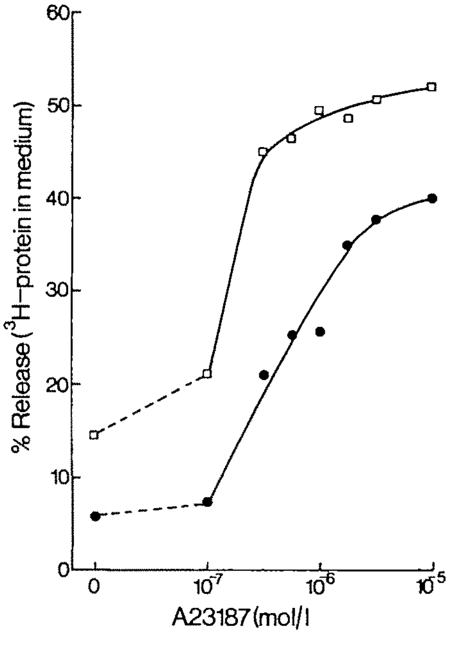


**Figure 6** The effect of A-23187 on the release of $^3$H-labelled protein from rat exocrine pancreas in the absence (●) and presence (□) of dibutyryl cyclic AMP ($10^{-3}$ mol/litre).

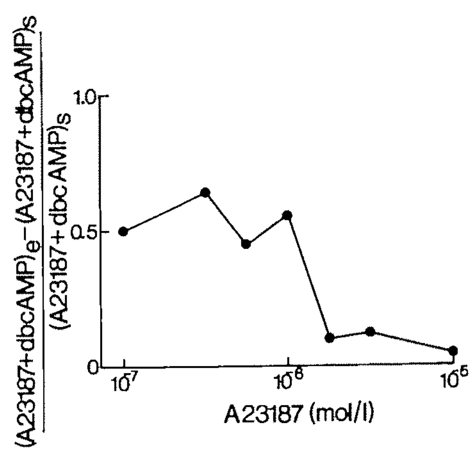


**Figure 7** The degree of potentiation of A-23187-stimulated $^3$H-protein release from rat pancreas by dibutyryl cyclic AMP (dbcAMP) as a function of A-23187 concentration in presence of [$Ca^{2+}$] $10^{-3}$ mol/litre.

particularly $Ca^{2+}$, across these membranes (Reed & Lardy, 1972). The effects of A-23187 on protein secretion from the exocrine pancreas was studied; results are shown in Figure 6. EGTA ($5 \times 10^{-5}$ mol/l) was present during both the 60 min chase and the 60 min incubation with the stimulus.

As the ionophore is insoluble in water, it was dissolved in 100% ethanol and diluted 1:100 with incubation buffer. Ethanol (1%) did not affect control release of $^3$H-protein nor radioactive protein release caused by $10^{-3}$ mol/l dibutyryl cyclic AMP alone. Protein release caused by A-23187 or A-23187 plus dibutyryl cyclic AMP was maximal at a calcium concentration of $10^{-3}$ mol/l (see Figure 8). At this concentration of calcium, the stimulatory effect (% total $^3$H-protein released minus % total $^3$H-protein released in control) of $5 \times 10^{-7}$ mol/l A-23187 was 8% and that of $5 \times 10^{-6}$ mol/l A-23187 was 21%. The stimulatory effect of dibutyryl cyclic AMP plus $5 \times 10^{-7}$ mol/l A-23187 was 25% at $10^{-3}$ mol/l $Ca^{2+}$; that of dibutyryl cyclic AMP plus $5 \times 10^{-6}$ mol/l A-23187 was 35%. The stimulatory effect of dibutyryl cyclic AMP alone at $10^{-3}$ mol/l $Ca^{2+}$ was 8%. Thus the experimental value for the stimulatory effect of dibutyryl cyclic AMP plus either concentration of A-23187 was approximately 1.5 times the sum of the individual stimulatory effects of the two agents, i.e. dibutyryl cyclic AMP potentiated the secretory effect of A-23187. The degree of potentiation as a function of A-23187 concentration was calculated and the results illustrated in Figure 7. In contrast to that seen when carbachol was the secretagogue (Figure 3) the degree of potentiation of A-23187 dependent secretion by dibutyryl cyclic AMP was maximal and constant (approximately 0.5) at low secretagogue concentrations ($10^{-7}$ to $10^{-6}$ mol/l) and decreased rapidly to a minimal value of 0.045 as the concentration of A-23187 was increased from $10^{-6}$ to $10^{-5}$ mol/litre.

The effect of A-23187 on $^3$H-protein release from pancreas pieces as a function of $Ca^{2+}$ concentration is illustrated in Figure 8. When extracellular $Ca^{2+}$ concentration was less than $10^{-4}$ mol/l A-23187 ($5 \times 10^{-7}$ or $5 \times 10^{-6}$ mol/l) had no effect on $^3$H-protein release. As the extracellular $Ca^{2+}$ concentration increased A-23187-dependent release of $^3$H-protein increased to a maximum at $10^{-3}$ mol/l $Ca^{2+}$. This concentration of $Ca^{2+}$ was optimum for both concentrations of A-23187 tested ($5 \times 10^{-7}$ and $5 \times 10^{-6}$ mol/litre). The potentiation of A-23187-dependent protein release by dibutyryl cyclic AMP was also $Ca^{2+}$-dependent and optimum at $10^{-3}$ mol/l $Ca^{2+}$. Using the data in Figure 8 the degree of potentiation was calculated to determine whether it was a function of the concentration of A-23187 and/or calcium. The results are illustrated in Figure 9. In contrast to carbachol (see Figure 2) the degree of potentiation of A-23187-dependent protein release decreased when the concentration of the ionophore was increased; this occurred at $5 \times 10^{-4}$ or $10^{-3}$ mol/l $Ca^{2+}$. The degree of potentiation was also inversely related to the $Ca^{2+}$

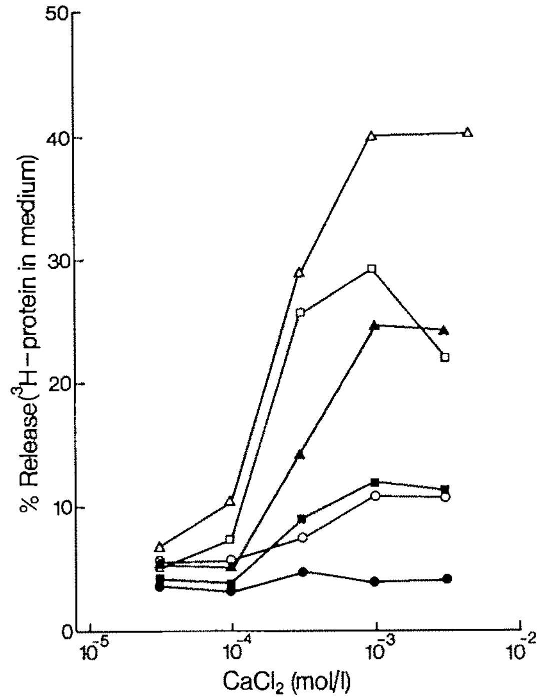


**Figure 8** The effect of A-23187 $5 \times 10^{-7}$ or $5 \times 10^{-6}$ mol/l, in the presence or absence of dibutyryl cyclic AMP $10^{-3}$ mol/l, on ³H-labelled protein release from rat pancreas pieces as a function of the concentration of extracellular calcium. EGTA ($5 \times 10^{-5}$ mol/l) and the desired concentration of calcium were present in the Krebs-Ringer bicarbonate buffer as indicated. The data are from one representative experiment: (●) 1% ethanol; (○) 1% ethanol+dibutyryl cyclic AMP $10^{-3}$ mol/l; (■) A-23187, $5 \times 10^{-7}$ mol/l; (□) A-23187, $5 \times 10^{-7}$ mol/l+dibutyryl cyclic AMP; (▲) A-23187, $5 \times 10^{-6}$ mol/l; (△) A-23187, $5 \times 10^{-6}$ mol/l+dibutyryl cyclic AMP.

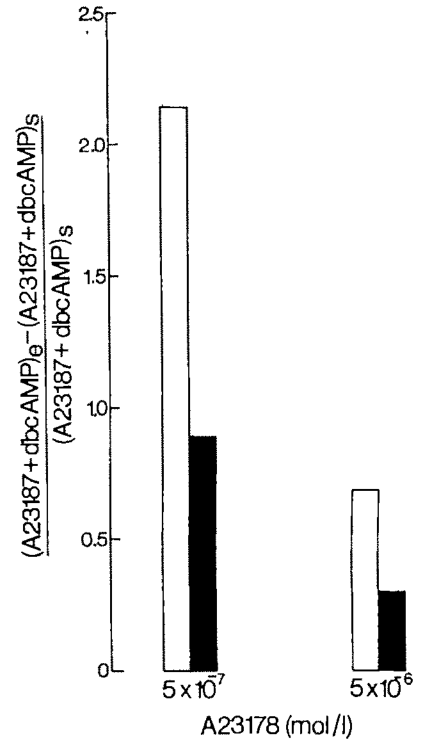


**Figure 9** The degree of potentiation of A-23187-stimulated ³H-protein release from rat pancreas by dibutyryl cyclic AMP (dbcAMP) at two [Ca$^{2+}$], $5 \times 10^{-4}$ mol/l (open columns) and $10^{-3}$ mol/l (closed columns).

concentration, i.e. as the Ca$^{2+}$ concentration was increased the degree of potentiation decreased.

## Discussion

The studies described in this paper were designed to elucidate the mechanism by which dibutyryl cyclic AMP potentiates the secretory effect of carbachol and specifically to test the hypothesis that dibutyryl cyclic AMP acts by maintaining intracellular calcium concentration at a level which is optimum for secretion. When protein secretion was stimulated by carbachol the absolute amount of radioactive protein released depended on the concentration of extracellular calcium as well as on the concentrations of carbachol and dibutyryl cyclic AMP. The degree of potentiation of the carbachol effect by dibutyryl cyclic AMP however, was independent of the concentration of carbachol at carbachol concentrations greater than $10^{-7}$ mol/l; that is, once the carbachol concentration was sufficient to stimulate secretion on its own the degree of potentiation depended only on the concentration of extracellular calcium and was inversely related to it. This relationship is predicted from the proposal that the limiting factor in carbachol stimulated secretion at all concentrations of carbachol is the cytoplasmic [Ca$^{2+}$] and that dibutyryl cyclic AMP acts by conserving and/or mobilizing intracellular Ca$^{2+}$.

Further evidence that dibutyryl cyclic AMP was acting by conserving intracellular Ca$^{2+}$ was obtained from the investigation of $^{45}$Ca flux between the tissue and incubation medium. Increased calcium influx into the acinar cell does not appear to be a major component of enzyme secretion initiated by cholinomimetic drugs (Matthews, Petersen & Williams, 1973), a conclusion which is supported by our results.

In our studies it was found that enzyme secretion from rat exocrine pancreas caused by carbachol or carbachol plus dibutyryl cyclic AMP was associated with an increased $^{45}$Ca efflux. Similar results have been reported by others (Case & Clausen, 1973; Heisler & Grondin, 1973; Matthews *et al.*, 1973). Although $^{45}$Ca efflux and enzyme secretion were stimulated in a similar dose-dependent fashion by carbachol, radioactive calcium efflux was not directly related to protein secretion; the greatest amount of protein release occurred with carbachol plus dibutyryl cyclic AMP but the largest efflux of $^{45}$Ca was caused by carbachol alone. This suggests that the zymogen granules are not the major or sole source of this $Ca^{2+}$ and thus implicates some other intracellular pool. These experiments also suggest that cholinomimetic drugs are able to mobilize $Ca^{2+}$ from some intracellular pool(s).

Dibutyryl cyclic AMP plus carbachol decreased the rate of $^{45}$Ca efflux to less than that caused by carbachol alone, and dibutyryl cyclic AMP alone decreased the rate of $^{45}$Ca efflux to less than control values. Dibutyryl cyclic AMP is thus able to conserve intracellular $Ca^{2+}$, and this action is independent of activation of the secretory process by carbachol.

Although the source of the increased intracellular calcium has not been firmly established for any secretory system, Borle (1973) showed that in many cells a likely source is the mitochondria. Because of the number of mitochondria and the rapid rate of movement of $Ca^{2+}$ across the mitochondrial membrane, this organelle can quickly increase or decrease the concentration of cytoplasmic ionized $Ca^{2+}$ in amounts which are believed necessary for physiological processes. Borle (1974) has also suggested that cyclic nucleotides may directly control uptake and release of $Ca^{2+}$ by mitochondria in some cells, thereby controlling the $Ca^{2+}$ concentration in the cytoplasm. In our experiments dibutyryl cyclic AMP cannot be only mobilizing $Ca^{2+}$ from intracellular stores since this would result in an increased rate of $^{45}$Ca efflux from the cell, whereas a decrease in $^{45}$Ca efflux is actually observed. The primary action of dibutyryl cyclic AMP, therefore, must be to maintain cytoplasmic ionized calcium by inhibiting efflux of $Ca^{2+}$ from the cytoplasm to the extracellular space and perhaps inhibiting 'efflux' from the cytoplasm into storage organelles. This suggests that the inability of dibutyryl cyclic AMP itself to stimulate secretion is due to the fact that this nucleotide does not promote $Ca^{2+}$ mobilization from intracellular stores but can only maintain an elevated cytoplasmic $Ca^{2+}$ concentration caused by some other mechanism.

This conclusion leads to the prediction that in any situation where the limiting factor in secretion is the cytoplasmic calcium concentration and where the secretagogue does not act by inhibiting those processes which remove calcium from the cytoplasm, dibutyryl cyclic AMP should potentiate the effect of the secretagogue. This prediction was tested with the ionophore A-23187 as the secretagogue. Reed & Lardy (1972) showed that this compound inserted itself into the mitochondrial membrane and acted as a carrier of divalent cations in the direction of the concentration gradient. Eimerl *et al.* (1974) have shown that this ionophore causes the release of amylase from exocrine pancreas and that this effect is dependent on extracellular $[Ca^{2+}]$. If the action of the ionophore is specific then one must conclude that simply increasing the intracellular concentration of calcium is sufficient to initiate the process of amylase secretion. The concentration of intracellular calcium is determined by the difference between the rate of movement of calcium into the cell and the rate of movement of calcium out of the cell. In the presence of A-23187 calcium influx is essentially a function of $[\text{A-23187}] \times [Ca^{2+}]_o$, and calcium efflux is the sum of $[\text{A-23187}] \times [Ca^{2+}]_i$ plus the contribution of the physiological calcium efflux processes. When the concentrations of ionophore and calcium are small the contribution of the physiological mechanisms of calcium extrusion ('Calcium pump') are maximized and therefore any agent which inhibits this pump would contribute maximally to maintaining a greater intracellular calcium concentration. The contribution of this 'pump' is minimized as the ionophore concentration increases and as the extracellular concentration of calcium is increased. It should be noted that the $Ca^{2+}$ 'pump' referred to here may be at the plasma membrane and/or at the membrane of any intracellular organelle. The only assumption is that it pumps $Ca^{2+}$ out of the cytoplasm. One would therefore predict that if dibutyryl cyclic AMP potentiates the action of secretagogues by inhibiting this $Ca^{2+}$ 'pump' it would maximally potentiate the effect of A-23187 at low ionophore and/or low extracellular $[Ca^{2+}]$ concentration and that the degree of potentiation would vary inversely as the concentration of ionophore and $Ca^{2+}$. This in fact is what was found: the potentiating effect of dibutyryl cyclic AMP is maximal at low ionophore and low extracellular $Ca^{2+}$ concentrations and almost totally disappears at a concentration of ionophore of $5 \times 10^{-6}$ mol/l and extracellular $[Ca^{2+}]$ of $10^{-3}$ mol/litre. These results are convincing support for the argument that in the rat exocrine pancreas an increase in cellular calcium is sufficient to initiate the secretion process and that dibutyryl cyclic AMP acts by inhibiting calcium efflux from the cytoplasm thus potentiating the action of secretagogues.

One striking difference between the interaction of dibutyryl cyclic AMP and ionophore is that at the optimum extracellular calcium concentration dibutyryl cyclic AMP continues to potentiate the effect of carbachol even at carbachol concentrations which are 10 × optimum whereas the dibutyryl cyclic

AMP potentiation of the A-23187 effect is almost nil at optimum [A-23187]. This suggests that under the influence of carbachol the maximum rate of accumulation of calcium into the cytoplasm is never sufficient to achieve a calcium concentration optimal for protein secretion, i.e. the 'cytoplasmic' calcium concentration is always the limiting factor in secretion when carbachol is the secretagogue.

It is tempting to postulate that cyclic AMP, which accumulates in various tissues in response to hormone stimulation, functions to maintain cytoplasmic calcium concentrations by a mechanism similar to that proposed for the dibutyryl derivative. However, because of the increasing number of reports of the divergent effects of endogenous cyclic AMP and exogenously administered analogues of that nucleotide such a conclusion is premature.

**References**

BENZ, L., ECKSTEIN, B., MATTHEWS, E.K. & WILLIAMS, J.A. (1972). Control of pancreatic amylase release *in vitro*: effects of ions, cyclic AMP, and colchisine, *Br. J. Pharmac.*, **46**, 66–77.

BORLE, A.B. (1973). Calcium metabolism at the cellular level. *Fedn Proc.*, **32**, 1944–1950.

BORLE, A.B. (1974). Cyclic AMP stimulation of calcium efflux from kidney, liver and heart mitochondria. *J. membrane Biol.*, **16**, 222–236.

CASE, R.M. & SCRATCHERD, T. (1972). The actions of dibutyryl cyclic adenosine 3′,5′-monophosphate and methyl xanthines on pancreatic exocrine secretion. *J. Physiol., Lond.*, **223**, 649–667.

CASE, R.M. & CLAUSEN, T. (1973). The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas. *J. Physiol., Lond.*, **235**, 75–102.

DOUGLAS, W.W. (1968). Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmac.*, **34**, 451–474.

EAGLE, H. (1959). Aminoacid metabolism in mammalian cell cultures. *Science*, **130**, 432–437.

EIMERL, S., SAVION, N., HEICHAL, O. & SELINGER, Z. (1974). Induction of enzyme secretion in rat pancreatic slices using the ionophore A-23187 and calcium. *J. biol. Chem.*, **249**, 3991–3993.

GOLDBERG, N.D., O'DEA, R.F. & HADDOX, M.K. (1973). Cyclic GMP. *Advances in cyclic nucleotide research*, **3**, 155–223.

HEISLER, S., FAST, D. & TENENHOUSE, A. (1972). Role of Ca$^{2+}$ and cyclic AMP in protein secretion from rat exocrine pancreas. *Biochim. Biophys. Acta*, **279**, 561–572.

HEISLER, S. & GRONDIN, G. (1973). Effect of lanthanum on $^{45}$Ca flux and secretion of protein from rat exocrine pancreas. *Life Sci.*, **13**, 783–794.

HOKIN, L.E. (1966). Effect of calcium omission on acetylcholine-stimulated amylase secretion and phospholipid synthesis in pigeon pancreas slices. *Biochim. biophys. Acta*, **115**, 219–221.

JAMIESON, J.D. & PALADE, G.E. (1967). Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the golgi complex. *J. cell. Biol.*, **34**, 577–596.

MATTHEWS, E.K., PETERSEN, O.H. & WILLIAMS, J.A. (1973). Pancreatic acinar cells: acetylcholine-induced membrane depolarization, calcium efflux and amylase release. *J. Physiol., Lond.*, **234**, 689–701.

RASMUSSEN, H. (1970). Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science*, **170**, 404–412.

RASMUSSEN, H., GOODMAN, D.B.P. & TENENHOUSE, A. (1972). The role of cyclic AMP and calcium in cell activation. *CRC Critical Reviews in Biochemistry*, **1**, 95–148.

RASMUSSEN, H. & TENENHOUSE, A. (1968). Cyclic adenosine monophosphate, Ca$^{2+}$, and membranes. *Proc. Natl Acad. Sci. U.S.A.*, **59**, 1364–1370.

REED, P.W. & LARDY, H.A. (1972). A-23187: a divalent cation ionophore. *J. biol. Chem.*, **247**, 6970–6977.

ROBBERECHT, P. & CHRISTOPHE, J. (1971). Secretion of hydrolases by perfused fragments of rat pancreas: effect of calcium. *Am. J. Physiol.*, **220**, 911–917.

ROBBERECHT, P., DESCHODT-LANCKMAN, M., DE NEEF, P., BORGEAT, P. & CHRISTOPHE, J. (1974). *In vivo* effects of pancreozymin, secretion, vasoactive intestinal polypeptide and pilocarpine on the levels of cyclic AMP and cyclic GMP in rat pancreas. *Fedn Eur. Biochem. Soc. Letters* **43**, 139–143.

WILLIAMS, J.A. & LEE, M. (1974). Pancreatic acinar cells: use of a Ca$^{2+}$ ionophore to separate enzyme release from the earlier steps in stimulus-secretion coupling. *Biochem. Biophys. Res. Commun.*, **60**, 542–548.

*(Received May 4, 1976.)*

*Br. J. Pharmac.* (1976), **58**, 613–620

# A COMPARISON OF AFFINITY CONSTANTS FOR MUSCARINE-SENSITIVE ACETYLCHOLINE RECEPTORS IN GUINEA-PIG ATRIAL PACEMAKER CELLS AT 29°C AND IN ILEUM AT 29°C AND 37°C

R.B. BARLOW, K.J. BERRY, P.A.M. GLENTON, N.M. NIKOLAOU & K.S. SOH
Departments of Pharmacology, Universities of Edinburgh and Bristol

1 The affinity of 17 compounds for muscarine-sensitive acetylcholine receptors in atrial pacemaker cells and ileum of the guinea-pig has been measured at 29°C in Ringer-Locke solution. Measurements were also made at 37°C with 7 of them.

2 Some of the compounds had much higher affinity for the receptors in the ileum than for those in the atria. For the most selective compound, 4-diphenylacetoxy-*N*-methylpiperidine methiodide, the difference was approximately 20-fold. The receptors in the atria are therefore different in structure from those in the ileum.

3 The effects of temperature on affinity are not the same for all the compounds tested, indicating different enthalpies and entropies of adsorption and accounting for some of the difficulty experienced in predicting the affinity of new compounds.

## Introduction

From measurements of the affinity constants of a number of compounds for muscarine-sensitive acetylcholine receptors in the ileum, bronchial muscle and iris of the guinea-pig, Barlow, Franks & Pearson (1972) concluded that the receptors were indistinguishable. Estimates of the affinity constant of a compound were not significantly different for the three types of tissue and, when allowance is made for the effects of different recording conditions on the estimates of affinity constants (Butt, 1972), any differences in receptor structure, which may exist but cannot be detected, must be small. Although Arunlakshana & Schild (1959) obtained results with mepyramine and diphenhydramine which suggested that histamine receptors in ileum and respiratory tract were similarly indistinguishable, the findings are surprising in view of the different time-course of the responses to the agonist; bronchial muscle contracts very much more slowly than the ileum. They are also surprising in view of the marked differences which are found in nicotine-sensitive receptors both in the specificity of blocking agents and in the stereospecificity of nicotine itself (Barlow & Hamilton, 1965).

The present work describes an attempt to see whether the muscarine-sensitive acetylcholine receptors of the guinea-pig atrial pacemaker cells are also indistinguishable from those of the ileum. There were three reasons for choosing this preparation. First, because the agonist causes hyperpolarization in this tissue, compared with depolarization in the other preparations. Second, because of the discovery that there are differences among types of histamine receptor and that the $H_2$-receptors occur in atria (Black, Duncan, Durant, Ganellin & Parsons, 1972). Third, because of the possible practical importance of drugs which would selectively block muscarine-sensitive acetylcholine receptors in the ileum more than those in the heart.

For testing the actions of drugs on isolated atria it has been customary to work at 29°C and to use Ringer-Locke solution (Burn, 1952). We attempted to work at 37°C but found the responses to carbachol became erratic after an hour or two, so we reverted to using 29°C. We therefore also made measurements on the ileum at this temperature and with the same physiological salt solution. The values on the ileum were different from those previously obtained at 37°C in Tyrode solution and the effect of temperature appeared to be different for different compounds. This suggested that it might be possible to obtain some idea of the enthalpy of adsorption (ΔH) of the antagonist because

$$\frac{\Delta \ln K}{\Delta \frac{1}{T}} = \frac{-\Delta H}{R}$$

and hence to assess entropies of adsorption, $\Delta S$, because

$$\ln K = \frac{-\Delta G}{RT}$$

and

$$T\Delta S = \Delta H - \Delta G$$

We therefore made measurements with some of the compounds at 37°C in Ringer-Locke solution.

The work falls into two parts. In the first, measurements were made with 10 compounds on the ileum and atria at 29°C in Ringer-Locke solution and, in the second part, measurements were also made simultaneously with the ileum at 37°C. In the first part comparisons were made of values of log K, as in previous work (Barlow *et al.*, 1972), but it is possible that part of the error attached to them may be associated with the choice of the concentrations of antagonist tested, if the compounds are not behaving strictly competitively. Some antagonists are known which produce results consistent with competition at low concentrations but which are not competitive at higher concentrations (Abramson, Barlow, Mustafa & Stephenson, 1969; Lüllmann, Ohnesorge, Schauwecker & Wasserman, 1969; Mitchelson, 1975). Although the compounds were tested in concentrations in which they seemed to be acting competitively, because the results obtained with higher concentrations were reasonably in agreement with those obtained with lower concentrations, it seemed desirable to perform a second group of experiments in which any possible bias was eliminated by testing the compounds at exactly the same concentration on the two types of preparation and comparing the dose-ratios.

## Methods

The guinea-pig ileum and atria were suspended in Ringer-Locke solution and aerated with $O_2$ (Edinburgh Staff, 1972). In the first group of experiments hexamethonium was present in a concentration of $3 \times 10^{-4}$M; in the second group the concentration was $2.76 \times 10^{-4}$M, except in one set in which the hexamethonium was omitted. The contractions of the ileum were recorded isotonically. Those of the atria were recorded in the first group of experiments with a strain-gauge (load about 0.2 g) and a Devices pen-recorder and in the second group with a very light spring-loaded transducer, made by Mr R.O. Morris, connected to a Vitatron potentiometric recorder.

Drug and wash solutions were applied automatically. Carbachol was the agonist except in a few experiments with acetyl-$\beta$-methylcholine. For the atria the agonist was given once every 16 min and allowed to act for 3 min in the first group of experiments and for 4 min in the second group, and then washed out. The preparation was washed again 5 min later. The time required for 50 beats was measured with a stopwatch at frequent intervals and the effect of the agonist was expressed as the percentage increase in time, calculated from the value at the end of the application of the agonist and the value just before its application. The size of the contractions was nearly always reduced much more than the rate and the dose-effect curves were usually steep; quite a small increase in concentration was sufficient to increase the effect from slight slowing to apparent arrest of the atria, with any contractions being too small to be counted. After a control period in which responses were obtained with low and high doses of agonist (often 2 and $4 \times 10^{-7}$M carbachol) the preparation was exposed to the concentration of antagonist and responses obtained with increased concentrations of agonist. Because of the long time interval necessary between doses of agonist, the calculation of the dose-ratio was usually based on two pairs of control responses (to low and high concentrations of agonist) and two pairs after the action of the antagonist.

The experiments on the ileum were performed exactly as described previously, with the agonist allowed to act for 30 s and given once every 90 s (Barlow, Franks & Pearson, 1973). The concentration of agonist needed to obtain suitable control responses (often 1 and $2 \times 10^{-7}$M) was similar to that used in the experiments with the atria.

With some compounds the responses of the ileum to the agonist in the presence of the antagonist became regular within a few minutes but with the more potent compounds tested in very dilute solution it was often necessary to wait 20 min and even up to 40 min in some instances. A similar time interval was allowed in the experiments on the atria. Usually it seemed to be sufficient to ignore the first response obtained in the presence of the antagonist (after about 12 min exposure) and to use subsequent responses (after about 28 min exposure) which seemed to be regular, but with the most potent compounds the interval was lengthened to 40 minutes.

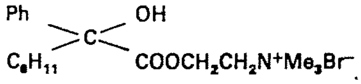
Each antagonist was tested in the same concentration on the ileum as on the atria and usually the same dose-ratio was tried initially. With nearly all the compounds it was clear that the dose-ratios were different for the two preparations and the concentrations of agonist were altered in order to measure the dose-ratio as accurately as possible. The initial dose-ratio tested on the ileum at 37°C in the second group of experiments was calculated from the published values of log K (in Tyrode solution) and subsequently adjusted when necessary. In the first group of experiments each antagonist was tested in at least two concentrations and the dose-ratios were used to calculate affinity constants. The result was expressed as the mean estimate of log K ± standard error based on the number of preparations used, as in previous work (Abramson *et al.*, 1969). In the second group of experiments only one concentration of each antagonist was tested. Mean estimates of the dose-ratio were calculated and the effect of changing temperature or changing tissue expressed as the ratio of the values of dose-ratio −1. The logarithm of this ratio should be the same as the difference in values of log K.

The temperatures at which the experiments were carried out, 29°C and 37°C, are subject to errors in measurement and control which may be as big as ±0.5°C.

*Compounds*

Carbachol, hexamethonium bromide and acetyl-β-methylcholine chloride were obtained from Koch-Light Ltd; the latter was recrystallized before use. Carbachol and hexamethonium bromide were also obtained from Sigma Chemical Co. Ltd. Atropine sulphate and hyoscine hydrobromide were obtained from BDH Ltd. The other antagonists were samples for which analyses have already been published

**Table 1** Values of the mean estimate of log K for guinea-pig atria and ileum are shown with the standard error and number of estimates. The agonist was carbachol, except where indicated and the Ringer-Locke solution contained hexamethonium ($3 \times 10^{-4}$M). Results for the ileum at 37°C are from previous work in Tyrode solution. Differences between values of log K for the ileum and atria are indicated by $\Delta_1$ and between values at 29° and 37°C by $\Delta_2$

| | *Atria* | *Ileum* | | *Ileum* | |
|---|---|---|---|---|---|
| | *29°C* | *29°C* | $\Delta_1$ | *37°C* | $\Delta_2$ |
| 4-Phenylacetoxy-*N*-methyl-piperidine HCl | 5.355<br>±0.019<br>(4) | 5.572<br>±0.008<br>(7) | 0.217 | 5.586 | −0.014 |
| $\mathrm{Ph_2CHCH_2OCH_2CH_2N^+Me_3Br^-}$ | 6.087<br>±0.040<br>(4) | 6.194<br>±0.010<br>(5) | 0.107 | 6.413 | −0.219 |
| $\mathrm{Ph_2CHCH_2OCH_2CH_2N^+Et_3Br^-}$ | 6.529<br>±0.016<br>(4) | 6.376<br>±0.009<br>(7) | −0.153 | 6.374 | 0.002 |
| Diphenylacetyltropine HCl | 7.405<br>±0.013<br>(4) | 7.956<br>±0.017<br>(6) | 0.551 | 8.110 | −0.154 |
| 4-Diphenylacetoxy-*N*-methyl-piperidine HCl | 7.571<br>±0.019<br>(4) | 8.362<br>±0.027<br>(7) | 0.791 | 8.361 | 0.001 |
| Diphenylacetyltropine-methiodide | 7.948<br>±0.027<br>(4) | 8.665<br>±0.032<br>(6) | 0.717 | 8.669 | −0.004 |
| $\mathrm{Ph_2C(OH)COOCH_2CH_2N^+Me_3Br^-}$ | 8.120<br>±0.048<br>(5) | 8.932<br>±0.023<br>(5) | 0.812 | 8.511 | 0.421 |
| with acetyl-β-methyl-choline as agonist | 8.158<br>±0.045<br>(3) | 8.936<br>±0.031<br>(4) | | | |
| Atropine | 9.126<br>±0.044<br>(3) | 9.259<br>±0.051<br>(4) | 0.133 | 9.007 | 0.252 |
| Atropine methiodide | 8.776<br>±0.023<br>(5) | 9.531<br>±0.066<br>(6) | 0.755 | 9.454 | 0.077 |
|  $\mathrm{Ph(C_6H_{11})C(OH)COOCH_2CH_2N^+Me_3Br^-}$ | 9.034<br>±0.010<br>(3) | 9.576<br>±0.042<br>(9) | 0.542 | 9.482 | 0.094 |

(Abramson *et al.*, 1969; Barlow *et al.*, 1973; Barlow, Franks & Pearson, 1974).

## Results

The results of the first group of experiments are summarized in Table 1 which shows the mean estimate of log K, with the standard error and number of preparations, for the atria and ileum at 29°C in Ringer-Locke solution. Published values of log K at 37°C in Tyrode solution are also included for comparison. It would be expected that there would be a bigger error attached to the estimates on the atria because the dose-ratios are calculated from much

**Table 2** Values of the mean estimate of the dose-ratio are shown with the standard error and number of estimates in experiments where the concentration of the antagonist was tested on guinea-pig atria at 29° and on ileum at 29° and 37°C; weighted means (see text) and values derived from them are shown in italics. The agonist was carbachol and the Ringer-Locke solution contained hexamethonium (2.76 × $10^{-4}$ M) except where indicated. The column headed $\Delta_1$ shows the log of the ratio (dose-ratio − 1) on the ileum to (dose-ratio − 1) on the atria and $\Delta_2$ shows the log of the corresponding ratio for the ileum at 29° and 37°C

| | *Atria* | *Ileum* | | *Ileum* | |
|---|---|---|---|---|---|
| | *29°C* | *29°C* | $\Delta_1$ | *37°C* | $\Delta_2$ |
| (−)-Hyoscine $10^{-7}$ | 95.5 | 364 | 0.584 | 174 | 0.322 |
| | ±19.5 | ±42.9 | | ±26.9 | |
| | (6) | (8) | | (6) | |
| | *93.0* | *372* | *0.606* | *198* | *0.275* |
| (−)-Hyoscine methiodide | 77.8 | 660 | 0.933 | 138 | 0.682 |
| 2 × $10^{-8}$M | ±9.00 | ±78.5 | | ±25.2 | |
| | (5) | (7) | | (6) | |
| | *76.5* | *634* | *0.923* | *123* | *0.715* |
| $Ph_2CHCH_2OCH_2CH_2N^+Me_3Br^-$ | 14.6 | 14.7 | 0.003 | 14.6 | 0.003 |
| 5 × $10^{-6}$M | ±0.74 | ±2.06 | | ±0.95 | |
| No hexamethonium | (5) | (6) | | (5) | |
| | *14.6* | *13.4* | *−0.040* | *14.6* | *0.040* |
| with hexamethonium | | 12.0 | | 9.41 | 0.117 |
| | | ±1.54 | | ±0.27 | |
| | | (3) | | (3) | |
| | | *11.5* | | *9.44* | *0.095* |
| $Ph_2CHCOOCH_2CH_2N^+Me_3Br^-$ | 25.6 | 47.9 | 0.280 | 44.1 | 0.037 |
| 5 × $10^{-7}$M | ±4.52 | ±3.00 | | ±4.05 | |
| | (5) | (5) | | (5) | |
| | *23.2* | *48.2* | *0.328* | *44.1* | *0.039* |
| 4-Diphenylacetoxy-*N*-methyl- | 7.28 | 132 | 1.319 | 63.3 | 0.323 |
| piperidine methiodide | ±1.10 | ±5.44 | | ±7.22 | |
| $10^{-7}$M | (4) | (6) | | (7) | |
| | *7.52* | *132* | *1.303* | *62.1* | *0.331* |
| repeated | 5.02 | 72.8 | 1.252 | 49.9 | 0.167 |
| | ±0.12 | ±2.07 | | ±3.35 | |
| | (3) | (4) | | (4) | |
| | *4.98* | *73.2* | *1.259* | *49.8* | *0.170* |
| 4-Benzilloyloxy-*N*-methyl- | 10.0 | 36.4 | 0.595 | 50.8 | −0.148 |
| piperidine methiodide | ±1.29 | ±5.26 | | ±8.67 | |
| $10^{-8}$M | (4) | (6) | | (5) | |
| | *10.0* | *36.8* | *0.600* | *48.2* | *−0.120* |
| 3-Diphenylacetoxy- | 43.8 | 89.4 | 0.315 | 61.1 | 0.168 |
| quinuclidine methiodide | ±7.69 | ±10.4 | | ±4.55 | |
| $10^{-8}$M | (4) | (5) | | (6) | |
| | *42.6* | *90.6* | *0.333* | *61.8* | *0.168* |
| Diphenylacetyl-pseudo- | 27.6 | 91.1 | 0.530 | 65.4 | 0.146 |
| tropine methiodide | ±1.65 | ±6.20 | | ±3.77 | |
| 5 × $10^{-7}$M | (4) | (7) | | (8) | |
| | *27.6* | *90.9* | *0.529* | *66.6* | *0.137* |

smaller numbers of responses than in the experiments with the ileum. There are systematic errors which exceed the statistical errors in this type of experiment (Abramson *et al.*, 1969) but it seems unlikely that the error attached to the mean estimates of log K on the atria is greater than 0.15 log units, compared with 0.1 log units on the ileum. In separate experiments the mean estimate of log K for diphenylethoxyethyltrimethylammonium bromide was calculated to be 6.421 (3), compared with 6.529 in Table 1 and mean estimates for methylatropinium were 8.872 (2) and 8.592 (4) compared with 8.776.

The results suggested that there were differences between the affinity for the muscarine-sensitive receptors in the atria and ileum, greater than could be accounted for by possible experimental errors. They also suggested that the effects of temperature on affinity were different for different compounds and that it would be desirable to make measurements at the two different temperatures with compounds tested simultaneously in the same physiological salt solution. The dose-ratios obtained in the second group of experiments are summarized in Table 2. In calculating the mean it seemed that the error might be reduced by weighting the results depending on the correction which had been made to calculate the matching dose-ratio from that actually tested. The values were therefore weighted according to the square of this correction factor (expressed as a number less than 1): for a perfect match between responses in the presence of the antagonist and responses in its absence the weight was one. For a poor match in which the high concentration in the presence of the antagonist matched the low concentration in its absence (so the calculated dose-ratio is exactly twice that actually tested), or with the low concentration in the presence of the antagonist matching the high concentration in its absence (so the calculated dose-ratio is exactly half that actually tested), the weight was only 0.25. The weighted means so obtained, however, did not differ greatly from the unweighted ones, nor did the derived values, $\Delta_1$ and $\Delta_2$ (see Table 2).

The individual dose-ratios were used to calculate log K as in the previous work and the differences between the mean values of log K also gave values of $\Delta_1$ and $\Delta_2$ which were very close to those calculated from the log of the ratios of (dose-ratio −1). For example the values of $\Delta_1$ for hyoscine were 0.584 from values of mean dose-ratios, 0.606 from values of weighted means dose-ratios, and 0.587 from the difference between mean values of log K.

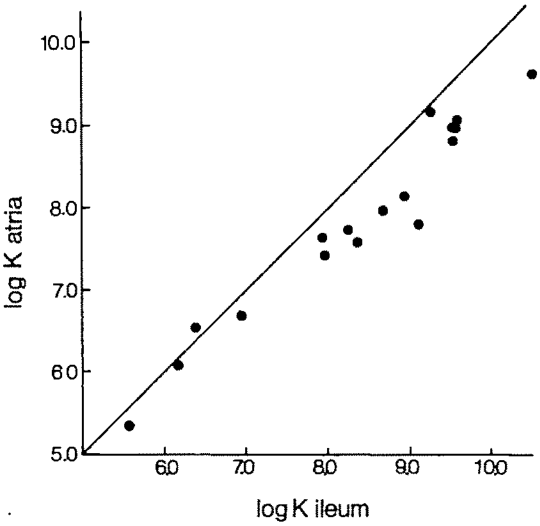


**Figure 1** Mean values of log affinity constant for the muscarine-sensitive receptors in the guinea-pig atria plotted against those for the receptors in the ileum measured at the same temperature (29°C) and in the same physiological salt solution (Ringer-Locke solution). If the affinity were the same for both receptors, the results should lie along the straight line.

From the repetition of some of the tests, however, it is clear that there remain large experimental errors but the differences between the affinity of many of the compounds for the receptors in the atria and the ileum at 29°C are much greater than these. Although the compounds selected nearly all had higher affinity for the ileum than for the atria (Figure 1), the difference does not appear to depend only on affinity for the ileum so could not be explained as arising simply from some bias associated with measuring high affinity. The most selective compound, 4-diphenylacetoxy-methylpiperidine methiodide, which has only about 1/20th the affinity for the receptors in the atria that it has for those in the ileum, is actually slightly weaker than atropine on the ileum.

There is much less certainty about differences in the effects of temperature on affinity. Estimates of log K at 29°C are probably subject to greater errors than those at 37°C, partly because of the slower response of the muscle and partly because of poorer control of temperature. Values of $\Delta_2$ listed in Table 1 are of very doubtful significance because they were not obtained from experiments made in the same physiological salt solution. The mean value of $\Delta_2$ for the results in Table 2 is $0.20 \pm 0.076$ (s.e.; 9 results) so it seems probable that the values for hyoscine methiodide and for 4-benziloyloxy-*N*-methylpiperidine methiodide are significantly different from the others. There does not seem to be any reason for believing that the compounds all have the same temperature coefficient.

## Discussion

In experiments on the effects of antagonists on the inotropic actions of carbachol on guinea-pig atria in

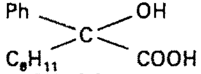
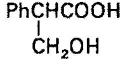
Tyrode solution at 30°C, Lüllman *et al.* (1969) obtained dose-ratios of 127 and 135 with $10^{-7}$M atropine (log $K = 9.10$, 9.13) and 180 with $8 \times 10^{-8}$M methylatropinium (log $K = 9.35$). Although the value for atropine is similar to that obtained in this work (9.126), the value for methylatropinium is appreciably higher than ours (8.776) and closer to that expected for the ileum (9.531). This suggests that the receptors at which carbachol acts to affect the rate of beating are different from those at which it acts to decrease the force of contraction. Alternatively it must be supposed that we have in some way underestimated the affinity of particular compounds for the receptors in the atrial pacemaker cells, in spite of all our attempts to avoid any bias, which include using a longer time-cycle and agonist contact time than Lüllman *et al.* (1969; details given by Mitchelson, 1975, indicate a 7 min cycle with 3 min contact with agonist compared with our 16 min cycle). As the affinity of the enantiomers dexetimide and levetimide for the inotropic carbachol receptor has been measured by Gray, Lüllman, Mitchelson & Reil (1976) and there is very marked stereospecificity it would seem well worthwhile testing these compounds against the chronotropic actions of carbachol.

It would be expected that the specificity of compounds for different forms of the muscarine-sensitive acetylcholine receptors would be related to their chemical structure. As can be seen from Table 3, in the four instances studied the quaternary salts all had relatively higher affinity for the receptors in the ileum, but the extent is variable, being greater in atropine than in diphenylacetyltropine. Specificity for the ileum seems also to be increased by changes involving the introduction of oxygen. For example, the diphenylacetyl ester of choline has a higher value (0.30) than the analogous ether (0.11; not included in Table 3); the benzilic ester has a higher value still (0.81). Likewise the scopines have higher values than their tropine analogues. The replacement of choline by 4-hydroxy-*N*-methylpiperidine greatly increases specificity but this is reduced with the more complex ring structures, tropine, pseudo-tropine and 3-hydroxyquinuclidine. The effects are not additive; 4-benziloyloxy-*N*-methylpiperidine methiodide has a lower specificity than its diphenylacetyl analogue even though it combines three features apparently favourable to specificity, a quaternary group, a benzilic group and the 4-hydroxy-*N*-methylpiperidine ring. It has higher affinity for both types of receptor but the replacement of diphenyl by benziloyl increases affinity for the receptors in the atria over 10-fold whereas affinity for those in the ileum is only increased just over 2-fold, hence the decline in specificity.

It is almost certain that hydrophobic interactions make a major contribution to the binding of these compounds to the muscarine-sensitive acetylcholine receptor but the results suggest that at the receptors in the ileum there may be an appreciable contribution from polar interactions in suitable circumstances. Although it is possible to find compounds with reasonable specificity for receptors in the ileum, it seems that it is much more difficult to obtain compounds with specificity for the atria. This may indicate that the receptors differ only in some extra feature present in the receptors in the ileum and absent from those in the atria, or it may merely indicate that a sufficient variety of compounds has not yet been tested.

The effects of temperature on affinity have been

**Table 3** Chemical structure and degree of specificity for muscarine-sensitive receptors in guinea-pig ileum compared with those in atria (29°C)

| *Acid* | *Base* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Choline* | N-*methylpiperidin-4-ol* | | *Tropine* | | *Pseudo tropine* | *Scopine* | | *3-Hydroxyquinuclidine* |
| | | *Base* | *MeI* | *Base* | *MeI* | *MeI* | *Base* | *MeI* | *MeI* |
| $PhCH_2COOH$ | | 0.22 | | | | | | | |
| $Ph_2CHCOOH$ | 0.30 | 0.79 | 1.32 | 0.55 | 0.72 | 0.53 | | | 0.32 |
| $Ph_2COHCOOH$ | 0.81 | | 0.58 | | | | | | |
| (±)  | 0.54 | | | | | | | | |
|  $PhCH(CH_2OH)COOH$ | | | | 0.13 | 0.75 | | 0.58 | 0.93 | |

The values shown, $\Delta_1$ in Tables 1 and 2, indicate the log of the ratio of the affinity constants; a value of 1.0 indicates that the affinity for the receptors in the ileum is 10 times that for the receptors in the atria. The tropic acid was racemic for the esters of tropine (atropine) but optically active for the esters of scopine ((—)-hyoscine).

examined by Paton & Rang (1966) who found that the affinity of lachesine for the muscarine-sensitive receptors in guinea-pig ileum was increased 1.3-fold by lowering the temperature from 37.5° to 30.5°C. Mustafa (1967) reported that log K for phenylacetoxyethyl-*N*- methylpiperidinium iodide was increased by 0.18 when the temperature was lowered from 37° to 27°C. These indicate very similar enthalpies of adsorption (ΔH), approximately —7 kcal (—29 kJ)/mol for lachesine and —7.8 kcal (—33kJ)/mol for the second compound. They differ greatly in their free energy of adsorption, however; for lachesine ΔG=—12.6 kcal (—53kJ)/mol whereas for the much weaker phenylacetoxyethyl compound ΔG=—7.4 kcal (—31 kJ)/mol. The corresponding changes in TΔS are +5.6 and —0.4 kcal/mol.

If compounds have similar enthalpies of adsorption, changes in free energy of adsorption must be due to changes in the entropy of adsorption. Though there may be situations in which binding is likely to be associated with increased disorder, there are other situations where the binding process might be expected to increase order in the system. It therefore seems unlikely that the enthalpy of adsorption will be the same for all compounds. It is certainly known that it is different for the binding of inhibitors to acetylcholinesterase (Belleau, Tani & Lie, 1965; Belleau & Lavoie, 1968) for which the effects of temperature on affinity can be measured more accurately and over a wider range than in experiments with guinea-pig ileum.

It would then be expected that values of $\Delta_2$ in Table 2 should not all be the same and that it would be worth calculating the values of ΔH and TΔS (Table 4). Although ΔG is known reasonably accurately, the values of ΔH are not accurate because the errors in $\Delta_2$ are large and must be multiplied by a large number. A difference in log K in these experiments of 0.1 corresponds to a value of ΔH=5.3 kcal/mol: estimates of TΔS could well be incorrect by this amount. The calculations do suggest, however, that the entropy changes associated with binding are different for different compounds. In some instances there appears to be a moderate increase in entropy but with hyoscine methiodide it appears that the order in the system increases. Further results are clearly desirable, particularly as the present experiments were made in Ringer-Locke solution (which does not contain $Mg^{2+}$) instead of the more usual Tyrode or Krebs solution.

The independence of the enthalpy and entropy terms accounts for the difficulty of predicting affinity accurately. As Abramson *et al.* (1974) have remarked 'To predict the affinity of new compounds, then, the real problem is to predict the extent to which the effects of the various substituents fall short of the maximum' and changes in structure which lead to increases in overall order, so that TΔS is negative, will make ΔG correspondingly more positive and tend to reduce affinity. To predict affinity successfully it will therefore be necessary to have some idea of the effects of substituents on either the enthalpy or entropy of binding and this information cannot be obtained from measurements at only one temperature.

We thank the Wellcome Trust for support. K.S.S. was on leave from the Department of Pharmacology, University of Malaysia, Kuala Lumpur.

**Table 4** Estimates of the enthalpies and entropies of adsorption for compounds to muscarine-sensitive acetylcholine receptors in the guinea-pig ileum calculated from the results shown in Table 2

| | *ΔG* | *ΔH* | *TΔS* |
|---|---|---|---|
| (—)-Hyoscine | −13.1 | −17.1 | −4.0 |
| (—)-Hyoscine methiodide | −14.0 | −36.4 | −22.4 |
| $Ph_2CHCH_2OCH_2CH_2N^+Me_3Br^-$ | −8.7 | −6.4 | +2.3 |
| Diphenylacetylcholine bromide | −9.7 | −2.1 | +7.6 |
| 4-Diphenylacetoxy-*N*-methyl-piperidine methiodide | −12.5 | −17.1<br>−9.1* | −4.6<br>+3.4* |
| 4-Benziloyloxy-*N*-methyl-piperidine methiodide | −13.5 | +8.0 | +21.5 |
| 3-Diphenylacetoxy-quinuclidine methiodide | −10.9 | −9.1 | +1.8 |
| Diphenylacetyl-pseudo-tropine methiodide | −11.4 | −8.0 | +3.4 |

Values are in kcal/mol (multiply by 4.18 to convert to kJ/mol). The asterisk indicates the second set of results obtained with this compound. Note that the errors attached to the estimates of ΔH and TΔS may be as big as ± 5 kcal/mol.

## References

ABRAMSON, F.B., BARLOW, R.B., MUSTAFA, M.G. & STEPHENSON, R.P. (1969). Relationships between chemical structure and affinity for acetylcholine receptors. *Br. J. Pharmac.*, **37**, 207–233.

ABRAMSON, F.B., BARLOW, R.B., FRANKS, FIONA M. & PEARSON, J.D.M. (1974). Relationships between chemical structure and affinity for prostganglionic acetylcholine receptors of the guinea-pig ileum. *Br. J. Pharmac.*, **51**, 81–93.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48–58.

BARLOW, R.B., FRANKS, FIONA M. & PEARSON, J.D.M. (1972). A comparison of the affinities of antagonists for acetylcholine receptors in the ileum, bronchial muscle and iris of the guinea-pig. *Br. J. Pharmac.*, **46**, 300–314.

BARLOW, R.B., FRANKS, FIONA M. & PEARSON, J.D.M. (1973). Studies on the stereospecificity of closely related compounds which block postganglionic acetylcholine receptors in the guinea-pig ileum. *J. med. Chem.*, **16**, 439–446.

BARLOW, R.B. & HAMILTON, J.T. (1965). The stereospecificity of nicotine. *Br. J. Pharmac. Chemother.*, **25**, 206–212.

BELLEAU, B. & LAVOIE, J.L. (1968). A biophysical basis of ligand-induced activation of excitable membranes and associated enzymes. A thermodynamic study using acetylcholinesterase as a model receptor. *Canad. J. Biochem.*, **46**, 1397–1409.

BELLEAU, B., TANI, H. & LIE, F. (1965). A correlation between the biological activity of alkyltrimethylammonium ions and their mode of interaction with acetylcholinesterase. *J. Amer. Chem. Soc.*, **87**, 2283–2285.

BLACK, J.W., DUNCAN, W.A.M., DURANT, C.J., GANELLIN, C.R. & PARSONS, E.M. (1972). Definition and antagonism of histamine $H_2$-receptors. *Nature, Lond.*, **236**, 385–390.

BURN, J.H. (1952). *Practical Pharmacology*, p. 23, Oxford: Blackwell.

BUTT, ALISON A. (1972). The effects of different recording conditions on the estimates of affinity constants of antagonists for acetylcholine receptors in the guinea-pig ileum. *Br. J. Pharmac.*, **46**, 312–314.

EDINBURGH STAFF (1972). *Pharmacological Experiments on Isolated Preparations*. 2nd edition, pages 2, 58, 112. Edinburgh: Churchill Livingstone.

GRAY, J.A., LÜLLMANN, H., MITCHELSON, F. & REIL, G.-H. (1976). Stereoselective binding in cardiac tissue of the enantiomers of benzetimide, an antimuscarinic drug. *Br. J. Pharmac.*, **56**, 485–490.

LÜLLMANN, H., OHNESORGE, F.K., SCHAUWECKER, G.-C. & WASSERMANN, O. (1969). Inhibition of the actions of carbachol and DFP on guinea-pig ileum isolated atria by alkane-bis-ammonium compounds. *Eur. J. Pharmac.*, **6**, 241–247.

MITCHELSON, F. (1975). Antimuscarinic action of an alkane-bis-ammonium compound alone and in combination with (+)-benzetimide. *Eur. J. Pharmac.*, **33**, 237–246.

MUSTAFA, M.G. (1967). Ph.D. Thesis, University of Edinburgh, pp. 40–41.

PATON, W.D.M. & RANG, H.P. (1966). A kinetic approach to the mechanism of drug action. In *Advances in drug research* vol 3, eds., Harper, N.J. & Simmonds, Alma B., p. 65. London: Academic Press.

*(Received May 5, 1976.*
*Revised June 4, 1976.)*

# INDEX TO VOLUME 58

## AUTHOR INDEX

A

ABOULAFIA, JEANNINE, MENDES, GUN B., MIYAMOTO, MARIA E., PAIVA, A.C.M. & PAIVA, THEREZINHA B. Effect of indomethacin and prostaglandin on the smooth muscle contracting activity of angiotensin and other agonists, 223

ADOLPHE, M., DUNN, C.J., FONTAGNE, J., GIROUD, J.P., LECHAT, P., PAUBLANT, D. & WILLOUGHBY, D.A. Mitogenic activity of different types of non-immunological inflammatory exudates on macrophages in culture, 448P

ALGATE, D.R. & LEACH, G.D.H. The stimulated release of dopamine-β-hydroxylase from intact organs of normo- and hypertensive rats, 275P

ALRIC, R., LIGNON, F., LOUBATIERES, A.L., MANTEGHETTIT, M. & PUECH, R. Non-saturable co-operative binding of tolbutamide to isolated islets of Langerhans, 450P

ALTMAN, J., DA PONTE, F. & WORCEL, M. Altered reactivity of stomach fundus smooth muscle in Okamoto spontaneous hypertension, 432P

AMBROZI, L., BIRKMAYER, W., RIEDERER, P. & YOUDIM, M.B.H. L-DOPA and (−)-deprenil in treatment of Parkinson's Disease, 423P.

APPERLEY, EIRA, HUMPHREY, P.P.A. & LEVY, G.P. Receptors for 5-hydroxytryptamine and noradrenaline in rabbit isolated ear artery and aorta, 211

ARBUTHNOTT, G.W., GARCIA-MUNOZ, M., NICOLAOU, N.M., TULLOCH, I.F. & WRIGHT, A.K. (introduced by ASHCROFT, G.W.) Is the striato-nigral pathway responsible for 'feed-back' control of dopamine release?, 272P

ARMSTRONG, J.M. & LATTIMER, N. Prostaglandin $E_2$ released from the kidneys of genetic hypertensive rats contributes to vasoconstrictor supersensitivity to angiotensin II *in vitro*, 462P

ASHCROFT, G.W. *see* ARBUTHNOTT, G.W., 272P

AUCLAIR, M.C., JOURDON, P., LECHAT, P. & LEMEIGNAN, M. Action of cytochrome C on transmembrane potentials of normal or hypoxic guinea-pig myocardial strips, 431P

AZUMA, H., BANNO, K. & YOSHIMURA, T. Pharmacological properties of *N*-(3′,4′-dimethoxy-cinnamoyl)anthranilic acid (N-5′), a new anti-atopic agent, 483

B

BACH, CATHERINE S. & JASANI, M.K. Role of lymphocytes in accumulation of fibrin in rabbit skin homografts, 292P

BAKER, G.B., BERTOLLINI, A., DEL CARMINE, R., MARTIN, I.L. & RAITERI, M. Effects of *p*-chloro-β-phenylethylamine on uptake and release of putative amine neurotransmitters in rat brain, 420P

BANNO, K. *see* AZUMA, H., 483

BARBHAIYA, R.H. & TURNER, P. Fluorimetric determination of ampicillin and cephalexin, 473P

BARLOW, R.B., BERRY, K.J., GLENTON, P.A.M., NIKOLAOU, N.M. & SOH, K.S. Comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and in ileum at 29°C and 37°C, 613

BARTLETT, A.L. Actions of carbon tetrachloride, hexa-chloroethane and the products of their metabolism in sheep on *Fasciola hepatica*, 395

BAXTER, M.G., MILLER, A.A. & WHEATLEY, P.L. Comparative studies on the effects of metoclopramide and some known neuroleptics on the EEG of the conscious rat, 269P

BEAL, A.M. The effect of high rates of vasopressin administration on renal potassium and sodium excretion during potassium loading in the sheep, 81

BENDING, M.R., BENNETT, P.N. & ROWLAND, M. Pethidine pharmacokinetics in dog: dose and time studies, 472P

BENNETT, P.N., DAVIES, D.S. & HAWKESWORTH, GABRIELLE, M. *In vivo* absorption studies with paraquat and diquat in the dog, 284P

BENNETT, P.N. *see* BENDING, M.R., 472P

BERDEAUX, A., BOISSIER, J.R. & GIUDICELLI, J.F. Autonomous control of heart, myocardial blood flow and S-T segment in canine ischaemic myocardium, 410P

BERDEAUX, A., BOISSIER, J.R. & GIUDICELLI, J.F. Atenolol, regional myocardial blood flow and S-T segment in canine ischaemic myocardium, 411P

BERRY, K.J. *see* BARLOW, R.B., 613

BERTOLLINI, A. *see* BAKER, G.B., 420P

BEVAN, P., BRADSHAW, C.M. & SZABADI, E. The action of microelectrophoretically applied L-3,4-dihydroxy-phenylalanine (DOPA) on single cortical neurones, 239

BEVAN, P., BRADSHAW, C.M. & SZABADI, E. Neuronal responses to adrenoceptor agonists in cerebral cortex: evidence for excitatory α-adrenoceptors and inhibitory β-adrenoceptors, 418P

BIRDSALL, N.J.M., BRADBURY, A.F., BURGEN, A.S.V., HULME, E.C., SMYTH, D.G. & SNELL, C.R. Interactions of peptides derived from C-fragment of α-lipotropin with brain opiate receptors, 460P

BIRKMAYER, W. *see* AMBROZI, L., 423P

BISCOE, T.J., EVANS, R.H., HEADLEY, P.M., MARTIN, M.R. & WATKINS, J.C. Structure-activity relations of excitatory amino acids on frog and rat spinal neurones, 373

BLACK, A. KOBZA, GREAVES, M.W., HENSBY, C.N. & PLUMMER, N.A. A new method of obtaining human skin inflammatory exudate for pharmacological analysis, 317P

BLAKELEY, A.G.H. & NICOL, C.J.M. Accumulation of biogenic amines by rabbit erythrocytes, 294P

BLAU, K., GOODWIN, B., WOODS, H.F. & YOUDIM, M.B.H. Phenylethylamine formation in perfused rat liver, 474P

BOISMARE, F.R., HACPILLE, L., LEFRANCOIS, J.P. & LEPONCIN, M. Long-lasting effects of opening of blood-brain barrier on modifications induced by intracarotid injection of noradrenaline, 417P

BOISSIER, J.R., DUMONT, C., OBERLANDER, C. & PETERFALVI, M. Comparison of circling behaviour induced in nigro-striatal lesioned rats after peripheral administration of indole derivatives, 452P

BOISSIER, J.R., GODEFROY, F., SOUBRIE, P., THEBOT, M.H. & WEIL-FUGAZZA, J. Platelet uptake of $[^{14}C]$-5-HT in emotional and non-emotional rats, 454P

BOISSIER, J.R. *see* BERDEAUX, A., 410P

BOISSIER, J.R. *see* BERDEAUX, A., 411P

BOLAM, J.P., FORD-HUTCHINSON, A.W., SMITH, M.J.H. & WALKER, J.R. Sponge implantation test in rat as a model for screening anti-inflammatory activity, 472P

BOOT, J.R., DAWSON, W. & OSBORNE, D.J. Biological significance of prostaglandin-like substance released from immunologically challenged guinea-pig lungs, 471P

BOREHAM, P.F.L. & WRIGHT, I.G. Hypotension in rabbits infected with *Trypanosoma brucei*, 137

BOULU, R.G., LARSEN, R.G., PLOTKINE, M. & ROSSIGNOL, P. Effects of carbonic anhydrase inhibitors on cerebral cortex oxygen availability and resistance to hypoxia, 417P

BOURNE, R.C., FRITH, C.D., OWEN, F. & RILEY, G.J. (introduced by CROW, T.J.) The effect of subcutaneous injections of adrenaline on platelet MAO activity, 291P

BOWERY, N.G. Reversal of action of GABA antagonists by barbiturates, 456P

BOWMER, C.J. & LINDUP, W.E. Effect of endogenous metabolites on the binding of *o*-methyl red to human serum albumin, 283P

BRADBURY, A.F. *see* BIRDSALL, N.J.M., 460P

BRADSHAW, C.M. *see* BEVAN, P., 239

BRADSHAW, C.M. *see* BEVAN, P., 418P

BREUKER, E., DINGLEDINE, R. & IVERSEN, L.L. Evidence for naloxone and opiates as GABA antagonists, 458P

BRIATICO, G., GUISO, G., JORI, A. & RAVAZZANI, C. Effects of contraceptive agents on drug metabolism in various animal species, 173

BROADLEY, K.J. The release of a coronary vasodilator metabolite from the guinea-pig isolated perfused heart stimulated by catecholamines, histamine and electrical pacing and by exposure to anoxia, 89

BROWN, D.A., GARTHWAITE, J., HAYASHI, E. & YAMADA, S. Action of surugatoxin on nicotinic receptors in the superior cervical ganglion of the rat, 157

BROWN, F. & REDFERN, P.H. Studies on mechanism of action of amantadine, 561

BUDNY, J., DOW, R.C., ECCLESTON, D., HILL, A.G. & RITCHIE, ISOBEL, M. A comparison of D and L-tryptophan on the cerebral metabolism of 5-hydroxytryptamine and dopamine in the dog, 3

BUNCE, K.T., PARSONS, M.E. & ROLLINGS, NITA A. The effect of metiamide on acid secretion stimulated by gastrin, acetylcholine and dibutyryl cyclic adenosine 3′,5′-monophosphate in the isolated whole stomach of the rat, 149

BUNTING, S., HIGGS, G.A., MONCADA, S. & VANE, J.R. Generation of thromboxane $A_2$-like activity from prostaglandin endoperoxides by polymorphonuclear leukocyte homogenates, 296P

BURGEN, A.S.V. *see* BIRDSALL, N.J.M., 460P

BURKA, J.F., EYRE, P. & HOLROYDE, M.C. Dopaminergic (co-mediator) modulation of release of histamine and SRS-A in calf, 445P

C

CARDINAL, D.C. & HILL, T.W.K. (introduced by WALKER, J.M.G.) A device for controlling perfusion pressure, 463P

CARLTON, K.G. & SURGUE, M.F. Some effects of mazindol on the metabolism of monoamines in the rat brain, 271P

CHARLIER, R.H., RAUTHIER, J.A. & RICHARD, J.C. An indolizine with an amiodarone-like haemodynamic profile, 411P

CHEN, C-S., GATES, G.R. & REYNOLDSON, J.A. Effect of morphine and naloxone on priming-induced audiogenic seizures in BALB/c mice, 517

CHEVILLOTTE, E., DEVYNCK, M.A., MEYER, P., PAPADIMITRIOU, A., ROUZAIRE-DUBOIS, B. & WORCEL, M. Molecular mechanism of post-nephrectomy uterine supersensitivity to angiotensin, 433P

CHICHMANIAN, R.M., KINSUN, I., MOULIN, M.A. & SAVINI, E.C. Effect of glucocorticoids on turn-over of acetylcholine and prostaglandins in isolated guinea-pig ileum, 475P

CHRISTOPHER, L.J. *see* WEIR, R.D., 317P

CHUBB, I.W. & RAINE, A.E.G. (introduced by VAUGHAN WILLIAMS, E.M.) Long-term effects of propranolol on tyrosine hydroxylase and dopamine-β-hydroxylase in superior cervical ganglia of rabbit, 430P

CLANACHAN, A.S. & McGARTH, J.C. Effects of ketamine on the peripheral autonomic nervous system of the rat, 247

CLARK, A.L. & MITCHELSON, F. Inhibitory effect of gallamine on muscarinic receptors, 323

CLARKE, CORAL H., NICHOLSON, A.N. & STONE, BARBARA M. Effect of 1,5-benzodiazepines, clobazam and triflubazam on sleep of man, 439P

CLARKE, G. & LINCOLN, D.W. Central and peripheral inhibition of milk-ejection reflex: studies with β-adrenoceptor antagonists, 464P

COHEN, Y., LINDENBAUM, A., PORQUET, D. & WEPIERRE, J. Evidence of central cardiovascular effects of intracerebroventricular isoprenaline in anaesthetized rat, 435P

COLINA-CHOURIO, J., McGIFF, J.C., MILLER, M.P. & NASJLETTI, A. Possible influence of intrarenal generation of kinins on prostaglandin release from the rabbit perfused kidney, 165

COLLIER, H.O.J., HAMMOND, M.D. & SCHNEIDER, C. Effects of drugs affecting endogenous amines or cyclic nucleotides on ethanol withdrawal head twitches in mice, 9

COLLIER, H.O.J., McDONALD-GIBSON, WENDY J. & SAEED, S.A. Stimulation of prostaglandin biosynthesis by drugs: effects *in vitro* of some drugs affecting gut function, 193

COLQUHOUN, D. Analysis of end-plate current fluctuations produced by acetylcholine and acetylmonoethylcholine in rat muscle, 428P

CONOLLY, M.E., GREENACRE, J.K. & SCOFIELD, P. Dose-dependent desensitization of lymphocyte β-receptor function after long-term culture with isoprenaline, 448P

CONSTANTI, A. & NISTRI, A. Antagonism by some antihistamines of amino acid-evoked responses recorded from lobster muscle fibre and frog spinal cord, 583
CORBETT, A.D., MAIN, BARBARA, E., MUIR, T.C. & TEMPLETON, D. The receptors involved in catecholamine mediated growth and secretion in rat parotid gland, 281P
COTMAN, C.W., HAYCOCK, J.W. & LEVY, W.B. On the functional coupling of neurotransmitter uptake and release in brain, 569
COTTNEY, J., LEWIS, A.J. & NELSON, D.J. Arachidonic acid-induced paw oedema in the rat, 311P
COWAN, A., DETTMAR, P.W. & WALTER, D.S. The effect of acute doses of buprenorphine on concentrations of homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) in the rat forebrain, 275P
COX, B., FOZARD, J.R. & SMALL, R.C. Slide-tape programmes in the teaching of pharmacology, 315P
CREESE, R. & MACLAGAN, JENNIFER. Labelled decamethonium in cat muscle, 141
CROOKS, J. *see* WEIR, R.D., 317P
CROW, T.J. *see* BOURNE, R.C., 291P
CROW, T.J. *see* WADDINGTON, J.L., 453P
CURZON, G. & FERNANDO, J.C.R. Effect of aminophylline on tryptophan and other aromatic amino acids in plasma, brain and other tissues and on brain 5-HT metabolism, 533
CURZON, G. & MARSDEN, C.A. Effects of *p*-chlorophenylalanine and $\alpha$-methyltryptophan on rat social behaviour, 455P

D

D'MELLO, A. & KRUK, Z.L. Effective pharmacological demonstrations to 100 students, 341P
D'MELLO, A. *see* PILOT, MARIE-ANNE, 470P
DA PONTE, F. *see* ALTMAN, J., 432P
DALE, M. MAUREEN, EVINC, AKGÜN & VINE, PAULINE. The denervated cremaster muscle of the guinea-pig as a pharmacological preparation, 229
DALE, M. MAUREEN & MUID, R. A simple *in vitro* preparation of mammalian tissue exhibiting properties of slow tonic skeletal muscle, 288P
DAVE, K.C. & DHATTIWALA, A.S. Adrenoceptors of the guinea-pig urinary bladder, 37
DAVEY, A., MARRIOTT, A. & STOCKHAM, M.A. Fitting a general sigmoid model to pharmacological response curves, 289P
DAVIDSON, J.F. *see* WEIR, R.D., 317P
DAVIES, D.S. *see* BENNETT, P.N., 284P
DAVIES, J. & DRAY, A. Actions of enkephalin and morphine on spinal cord and brain stem neurones, 458P
DAWES, P. & REDFERN, P.H. A simple and inexpensive method for intracerebral administration of drug solutions to conscious rat, 464
DAWSON, W. *see* BOOT, J.R., 471P
DAY, M.D. & ROACH, A.G. Cardiovascular effects of dopamine after central administration into conscious cat, 505
DE BONO, D.P., GORDON, J.L., MACINTYRE, D.E. & PEARSON, J.D. Active transport of adenine and adenosine by platelets and cultured endothelial cells, 466P
DE JONG, W. & NIJKAMP, F.P. Centrally induced hypotension and bradycardia after administration of $\alpha$-methylnoradrenaline into area of nucleus tractus solitarii of rat, 593
DE POVER, A. & GODFRAIND, T. Role of potassium in inhibition by cardiac glycosides of $(Na^+-K^+)$-ATPase prepared from human heart, 413P
DEHPOUR, A.R., GHAFFARPOUR, F., HOSSEINZADEH, K. & KHOYI, M.A. (introduced by MICHAL, F.) Effect of denervation and cocaine on the response of isolated rat vas deferens to noradrenaline and methoxamine, 280P
DEL CARMINE, R. *see* DEL FIACCO, MARINA, *see* KOOB, G., 454P
DELAIGUE, R., DUCHENE-MARULLAZ, P., GUEORUIEV, G. & KANTELIP, J.P. Comparative effects of chloralose and pentobarbitone on atrioventricular conduction in dog, 408P
DELBARRE, B., SCHMITT, H. & SENON, DANIELLE. Effects of activation of $H_1$- and $H_2$-receptors on central cardiovascular structures in cats and on behaviour in chickens, 443P
DESOIZE, B., JARDILLIER, J.C., LEGER, C. & NAHAS, G.G. Delta-9-tetrahydrocannabinol and macromolecular synthesis, 419P
DETTMAR, P.W. *see* COWAN, A., 275P
DEVYNCK, M.A. *see* CHEVILLOTTE, E., 433P
DEWS, P.B. Effects of drugs on suppressed responding, 451P
DEY, P.K. & FELDBERG, W. Analgesia produced by morphine when acting from the liquor space, 383
DHATTIWALA, A.S. *see* DAVE, K.C., 37
DINGLEDINE, R. *see* BREUKER, E., 458P
DOW, R.C. *see* BUNDY, J., 3
DRAY, A. *see* DAVIES, J., 458P
DUBOST, M., ESCOUSSE, A., MOUNIE, J. & TRUCHOT, R. Peripheral effects of amphetamine-type anorectic drugs: inhibition of catecholamine-induced lipolysis, respiration, glucose utilization in adipose tissue of man and rat, 436P
DUCHENE-MARULLAZ, P. *see* DELAIGUE, R., 408P
DUCKWORTH, J.W., LANCE, J.W., LORD, G.D.A. & MYLECHARANE, E.J. (introduced by RAPER, C.) Histamine receptors in cranial circulation of monkey, 444P
DUMEUR, G., HUE, BERNADETTE, LWOFF, J.M., MOURIES, M.A. & TREMBLAY, D. Activity of anorectic drugs (amphetamine, amfepramone and UP507-04) on two models of obesity in animals, 437P
DUMONT, C. *see* BOISSIER, J.R., 452P
DUNN, C.J. *see* ADOLPHE, M., 448P
DUPUIS, B., GAUTIER, P., RICHER, G. & SERRE, M. A method for experimental study of arrhythmias following myocardial infarction in closed-chest dog. Its use as a test for anti-arrhythmic drugs, 409P

E

EAKINS, K.E., RAJADHYAKSHA, V. & SHROER, R. Prostaglandin antagonism by sodium *p*-benzyl-4-(1-oxo-2-(4-chlorobenzyl) - 3-phenylpropyl)phenylphosphonate (N-0164), 333
ECCLESTON, D. *see* BUNDY, J., 3
EFELLAH, M.S., MARSHALL, P.B. & TURNBULL, M.J. Effect of chronic pretreatment with sympathomimetic bronchodilator drugs on the responsiveness of guinea-pig lung adenylate cyclase *in vitro*, 274P

ERSKINE, Z.G. *see* WEIR, R.D., 317P
ESCOUSSE, A. *see* DUBOST, M., 436P
EVANS, R.H. & SMITH, J.W. The effect of catecholamines on the influx of calcium and the development of tension in denervated mouse diaphragm muscle, 109
EVANS, R.H. *see* BISCOE, T.J., 373
EVINC, AKGÜN *see* DALE, M. MAUREEN, 229
EYRE, P. *see* BURKA, J.F., 445P

F

FAGG, G.E., JORDAN, C.C. & WEBSTER, R.A. Release of endogenous amino acids from cat spinal cord *in vivo*, 440P
FARGES, J.P., FAUCON, G. & OLLAGNIER, M. Heterogeneous reactivity of atrial and ventricular cardiac contractile tissue, 409P
FARRAR, D.G. & VERITY, ANNE. Analysis of effects of isoxsuprine on guinea-pig atria and trachea, 431P
FAST, D. & TENENHOUSE, A. Effect of dibutyryl cyclic adenosine-3′,5′-monophosphate on protein secretion from rat exocrine pancreas *in vitro*, 605
FAUCON, G. *see* FARGES, J.P., 409P
FELDBERG, W. *see* DEY, P.K., 383
FERNANDO, J.C.R. *see* CURZON, G., 533
FERREIRA, S.H. & SMAJE, L.H. Bradykinin and functional vasodilatation in the salivary gland, 201
FILE, SANDRA E., HYDE, J. & POOL, M. (introduced by NEAL, M.J.) Effects of ethanol and chlordiazepoxide on social interaction in rats, 465P
FILLION, G., FILLION, M.P., JACOB, J. & ROUSSELLE, J.C. 5-HT and LSD high affinity binding sites to brain synaptosomal membranes, 425P
FILLION, M.P. *see* FILLION, G., 425P
FINCH, L. & HICKS, P.E. Studies on the marked anti-hypertensive properties of indapamide (SE 1520) in rats and cats, 282P
FIRRELL, J.C., PECK, M.J. & WILLIAMS, T.J. (introduced by LEWIS, G.P.) The polymorph transfer reaction: a model system for the study of acute inflammation, 310P
FLUDDER, JOAN M. & TONGE, SALLY R. (introduced by LEONARD, B.E.) Modification by oestrogen of effects of (+)-amphetamine sulphate on noradrenaline metabolism in discrete areas of rat brain, 422P
FONTAGNE, J. *see* ADOLPHE, M., 448P
FORD-HUTCHINSON, A.W. *see* BOLAM, J.P., 472P
FOSTER, R.W. How can one set multiple choice examinations of equal difficulty from year to year?, 305P
FOY, J.M. Learning from and attitudes to routine tape-slide teaching, 307P
FOY, J.M. & HOPKINS, D.J. Autonomic drugs and the eye, 315P
FOY, J.M. & McCURRIE, J.R. The blood pressure and respiration of the anaesthetized cat, 316P
FOY, J.M. & SENIOR, JUDITH. The teaching of pharmacology to undergraduate pharmacy students, 308P
FRY, J.M. & SENIOR, JUDITH. Teaching methods in pharmacology, 316P
FOZARD, J.R. & MOBAROK ALI, A.T.M. Inhibition of stimulant effect of 5-HT on cardiac sympathetic nerves by 5-HT and related compounds, 416P
FOZARD, J.R. & MOBAROK ALI, A.T.M. Evidence for tryptamine receptors on cardiac sympathetic nerves, 276P
FOZARD, J.R. *see* COX, B., 315P
FRITH, C.D. *see* BOURNE, R.C., 291P

G

GALLON, S.C. *see* WEIR, R.D., 317P
GARCIA-MUNOZ, M. *see* ARBUTHNOTT, G.W., 272P
GARDNER, C.R. & PHILLIPS, S.W. Heterogeneity of inhibitory mechanisms in the nucleus accumbens and preoptic area of the rat, 267P
GARNIER, D., GOUPIL, N., BARGEOT, J., POURRIAS, B. & ROUGIER, O. Effects of tryptamine on mechanical and electrical activities of sino-atrial frog auricular trabeculae, 410P
GARTHWAITE, J. *see* BROWN, D.A., 157
GATES, G.R. *see* CHEN, C-S., 517
GAUTIER, P. *see* DUPUIS, B., 409P
GHAFFARPOUR, F. *see* DEHPOUR, A.R., 280P
GEORGE, A.J. & JONES, G.T. Studies on monoamine oxidase in rat spinal cord, 279P
GIACHETTI, A. & HOLLENBECK, R.A. Extravesicular binding of noradrenaline and guanethidine in adrenergic neurones of rat heart: a proposed site of action of adrenergic neurone-blocking agents, 497
GIBSON, A. & JAMES, T.A. K$^+$-induced relaxations of the rat anococcygeus muscle, 305P
GIBSON, A., POLLOCK, D. & SPENCE, J.C. The effects of corticosteroids on the responses of the anococcygeus and gastrocnemius muscles to nerve stimulation in the pithed rat, 302P
GILLLESPIE, J.S. & TILMISANY, A.K. The action of tetraethylammonium chloride on the response of the rat anococcygeus muscle to motor and inhibitory nerve stimulation and to some drugs, 47
GIROUD, J.P. *see* ADOLPHE, M., 448P
GIUDICELLI, J.F. *see* BERDEAUX, A., 410P
GIUDICELLI, J.F. *see* BERDEAUX, A., 411P
GLENTON, P.A.M. *see* BARLOW, R.B., 613
GOADBY, P. & LITTLE, M. ANN. The action of metiamide in anaphylaxis *in vivo* in the guinea-pig, 303P
GODEFROY, F. *see* BOISSIER, J.R., 454P
GODFRAIND, T. *see* DE POVER, A., 413P
GOODWIN, B. *see* BLAU, K., 474P
GORDON, J.L. & MacINTYRE, D.E. Stimulation of platelets by bis-enoic prostaglandins, 298P
GORDON, J.L., MacINTYRE, D.E. & McMILLAN, R.M. Malondialdehyde production and the release reaction in rat blood platelets: inhibition by aspirin and indomethacin *ex vivo*, 299P
GORDON, J.L. & OVERMAN, H.J. Transport of 5-hydroxytryptamine by rat and human platelets, 300P
GORDON, J.L. *see* DE BONO, D.P., 466P
GOUPIL, D. *see* GARNIER, D., 410P
GREAVES, M.W. *see* BLACK, A. KOBZA, 317P
GREENACRE, J.K. *see* CONOLLY, M.E., 448P
GUEORUIEV, G. *see* DELAIGUE, R., 408P
GUISO, G. *see* BRIATICO, G., 173

H

HACPILLE, L. *see* BOISMARE, F.R., 417P
HAMMOND, M.D. *see* COLLIER, H.O.J., 9

HARICHAUX, P. & HARY, L. Some complementary data on AH 5158, an inhibitor of both $\alpha$- and $\beta$-adrenoceptors, 412P
HARY, L. *see* HARICHAUX, P., 412P
HAWKESWORTH, GABRIELLE M. *see* BENNETT, P.N., 284P
HAYASHI, E. *see* BROWN, D.A., 157
HAYCOCK, J.W. *see* COTMAN, C.W., 569
HEADLEY, P.M. *see* BISCOE, T.J., 373
HEDQVIST, P. Further evidence that prostaglandins inhibit release of noradrenaline from adrenergic nerve terminals by restriction of availability of calcium, 599
HENNINGSSON, S. & ROSENGREN, ELSA. Effect of nandrolone, an anabolic steroid on putrescine metabolism in mouse, 401
HENSBY, C.N. *see* BLACK, A. KOBZA, 317P
HICKS, P.E. *see* FINCH, L., 282P
HIGGS, G.A. *see* BUNTING, S., 296P
HILEY, C.R. Ontogenesis of muscarinic receptor sites in rat brain, 427P
HILL, A.G. *see* BUDNY, J., 3
HILL, R.C. & RÖMER, D. Comparison of the self-administration liabilities of morphine and pentazocine in the rhesus monkey, 270P
HILL, R.G. & PEPPER, C.M. Effects of morphine and met-enkephalin on nociceptive neurones in rat thalamus, 459P
HILL, T.W.K. *see* CARDINAL, D.C., 463P
HILLIER, K., ROBERTS, P.J. & WOOLLARD, P.M. Catecholamine-stimulated prostaglandin synthesis in rat brain synaptosomes, 462P
HÖGLUND, ELIN, WESTLING, H. & WHITE, T. Circulatory effects of oral and subcutaneous administration in normal subjects of a new bronchodilator, ibuterol, a prodrug to terbutaline, 43
HOLLENBRECK, R.A. *see* GIACHETTI, A., 497
HOLROYDE, M.C. *see* BURKA, J.F., 445P
HONERJAGER, P. & REITER, M. (introduced by TRENDELENBERG, U.) Batrachotoxin: activity-dependent prolongation of the cardiac action potential and positive inotropic effect, 415P
HOPKINS, D.J. *see* FOY, J.M., 315P
HOSSEINZADEH, K. *see* DEHPOUR, A.R., 280P
HOULT, J.R.S. Comparisons of performance in pharmacology examinations using multiple choice and essay questions, 306P
HOULT, J.R.S. Films in pharmacology teaching, 313P
HUE, BERNADETTE *see* DUMEUR, G., 437P
HULME, E.C. *see* BIRDSALL, N.J.M., 460P
HUMPHREY, P.P.A. *see* APPERLEY, EIRA, 211
HYDE, J. *see* FILE, SANDRA E., 465P

I

IDOWU, O.A. & ZAR, M.A. Inhibitory effect of clonidine on a peripheral adrenergic synapse, 278P
IMBS, J.L., SCHMIDT, M. & SCHWARTZ, J. Effect of (±)-propranolol on recovery of urinary concentration process after frusemide in rat, 413P
ISMAHAN, G., PARVEZ, H., PARVEZ, S. & RAZA-BUKHARI, A. Interrelations between MAO activity and carbohydrate metabolism, 450P
IVERSEN, L.L. *see* BREUKER, E., 458P
IVERSEN, SUSAN D. *see* KOOB, G., 454P

J

JACKSON, D.M. & RICHARDS, I.M. A further action of sodium cromoglycate, 301P
JACOB, J. *see* FILLION, G., 425P
JAMES, G.W.L. *see* MILLER, P., 442P
JAMES, T.A. *see* GIBSON, A., 305P
JARDILLIER, J.C. *see* DESOIZE, B., 419P
JASANI, M.K. *see* BACH, CATHERINE S., 292P
JENNER, P.G. & PYCOCK, C.J. Interaction of clonidine with dopamine-dependent behaviour in rodents, 469P
JETLEY, M. & WESTON, A.H. Some effects of D600, nifedipine and sodium nitroprusside on electrical and mechanical activity in rat portal vein, 287P
JHAMANDAS, K. & SUTAK, M. Morphine-naloxone interaction in the central cholinergic system: the influence of subcortical lesioning and electrical stimulation, 101
JONES, G.T. *see* GEORGE, A.J., 279P
JORDAN, C.C. *see* FAGG, G.E., 440P
JORI, A. *see* BRIATICO, G., 173
JOURDAN, P. *see* AUCLAIR, M.C., 431P

K

KALSNER, S. The lack of effect of oxytetracycline on responses to sympathetic nerve stimulation and catecholamines in vascular tissue, 261
KANTILEP, J.P. *see* DELAIGUE, R., 408P
KHOYI, M.A. *see* DEHPOUR. A.R., 280P
KINSUN, I. *see* CHICHMAANIAN, R.M., 475P
KIRBY, MARILYN J. & TURNER, P. Effect of some receptor antagonists on fenfluramine-induced glucose uptake into the isolated rat hemidiaphragm, 286P
KISIN, I.E. On the decrease of myocardial oxygen consumption induced by chloracyzine, 189
KOOB, G., DEL FIACCO, MARINA & IVERSEN, SUSAN D. Behavioural effects of EOS-induced changes in substantia nigra GABA levels, 454P
KRUK, Z.L., SMITH, L.A. & ZARRINDAST, M.R. Antagonism of responses to anorectics by selective receptor blockers, 468P
KRUK, Z.L. & ZARRINDAST, M.R. Mazindol anorexia is mediated by activation of dopaminergic mechanisms, 367
KRUK, Z.L. & ZARRINDAST, M.R. The effects of anorectic drugs on uptake and release of brain monoamines, 272P
KRUK, Z.L. *see* D'MELLO, A., 314P

L

LANCE, J.W. *see* DUCKWORTH, J.W., 444P
LARSEN, R.G. *see* BOULU, R.G., 417P
LATTIMER, N. *see* ARMSTRONG, J.M., 462P
LEACH, G.D.H. Developments in the use of television for pharmacology teaching, 314P
LEACH, G.D.H. Langendorff mammalian heart perfusion, 316P
LEACH, G.D.H. *see* ALGATE, D.R., 275P
LECHAT, P. *see* ADOLPHE, M., 448P
LECHAT, P. *see* AUCLAIR, M.C., 431P
LEFRANCOIS, J.P. *see* BOISMARE, F.R., 417P
LEGER, C. *see* DESOIZE, B., 419P

LEMEIGNAN, M. *see* AUCLAIR, M.C., 431P
LEONARD, B.E. *see* FLUDDER, JOAN M., 422P
LEPONCIN, M. *see* BOISMARE, F.R., 417P
LEVY, G.P. *see* APPERLEY, EIRA, 211
LEVY, W.B. *see* COTMAN, C.W., 569
LEWIS, A.J. *see* COTTNEY, J., 311P
LEWIS, D.A. & PARROTT, D.P. Effect of cortisol on $\alpha_1$-macroglobin and $\alpha_2$-acute phase globulin in arthritic rat, 411P
LEWIS, D.M. & WEHH, SANDRA N. Adrenaline on isotonic contractions of mammalian skeletal muscle, 467P
LEWIS, G.P. *see* FIRRELL, J.C., 310P
LEWIS, G.P. *see* WESTWICK, J., 297P
LIGNON, F. *see* ALRIC, R., 450P
LINCOLN, D.W. *see* CLARKE, G., 464P
LINDENBAUM, A. *see* COHEN, Y., 435P
LINDUP, W.E. *see* BOWMER, C.J., 283P
LITTLE, M. ANN *see* GOADBY, P., 303P
LORD, G.D.A. *see* DUCKWORTH, J.W., 444P
LOUBATIERES, A.L., RIBES, G. & VALETTE, G. Pharmacological agents and acute experimental hyperlactataemia in dog, 429P
LOUBATIERIES, A.L. *see* ALRIC, R., 450P
LUGNIER, C., MICHON, T. & STOCLET, J.C. Evolution of arotic and cardiac cyclic AMP phosphodiesterase during the onset of mineralocorticoid hypertension in rat, 434P
LULICH, K.M., MITCHELL, H.W. & SPARROW, M.P. The cat lung strip as an *in vitro* preparation of peripheral airways: a comparison of $\beta$-adrenoceptor agonists, autacoids and anaphylactic challenge on the lung strip and trachea, 71
LUNDELL, L., SVENSSON, S.E. & NILSSON, G. Further studies on the mode of action of isoprenaline on gastric secretion in the conscious rat, 17
LUSCOMBE, D.K. & NICHOLLS, P.J. Intersubject variability of sulphadimidine acetylation in student volunteers, 312P
LUSCOMBE, D.K. & NICHOLLS, P.J, Class experiment with student volunteers illustrating the influence of formulation on absorption of aspirin, 312P
LWOFF, J.M. *see* DUMEUR, G., 437P

M

MacINTYRE, D.E. *see* GORDON, J.L., 298P
MacINTYRE, D.E. *see* GORDON, J.L., 299P
MacINTYRE, D.E. *see* PEARSON, J.D., 466P
McCURRIE, J.R. *see* FOY, J.M., 316P
McDONALD-GIBSON, WENDY J., SAEED, S.A. & SCHNEIDER, C. Local antinociceptive and topical anti-inflammatory effects of propyl gallate in rodents, 573
McDONALD-GIBSON, WENDY J. *see* COLLIER, H.O.J., 193
McGRATH, J.C. *see* CLANACHAN, A.S.,247
McGIFF, J.C. *see* COLINA-CHOURIO, J., 165
McMILLAN, R.M. *see* GORDON, J.L., 299P
LAGAN, JENNIFER *see* CREESE, R., 141
MAIN, BARBARA E. *see* CORBETT, A.D., 281P
MANTEGHETTI, M. *see* ALRIC, R., 450P
MARCY, R. & QUERMONNE, M.A. Decremental skin conductance response in mice during iterative photostimulation: an attention-sustaining capacity model for psychopharmacological research, 437P
MARRIOTT, A. *see* DAVEY, A., 289P
MARSDEN, C.A. *see* CURZON, G., 455P
MARSHALL, P.B. *see* EFELLAH, M.S., 274P
MARSHALL, P.B. & STEVENSON, I.H. Career patterns in science graduate pharmacologists, 309P
MARSHALL, R.J. & PARRATT, J.R. Effects of dobutamine in early stages of acute myocardial infarction in dog, 407P
MARTIN, I.L. *see* BAKER, G.B., 420P
MARTIN, M.R. *see* BISCOE, T.J., 373
MENDES, GUN B. *see* ABOULAFIA, JEANNINE, 223
METCALF, G. & MYERS, R.D. Comparison between hypothermia induced by intraventricular injections of thyrotropin-releasing hormone, noradrenaline or calcium ions in unanaesthetized cats, 489
MEYER, P. *see* CHEVILLOTTE, E., 433P
MICHAL, F. *see* DEHPOUR, A.R., 280P
MICHON, T. *see* LUGNIER, C., 434P
MILLER, A.A. *see* BAXTER, M.G., 269P
MILLER, M.P. *see* COLINA-CHOURIO, J., 165
MILLER, P. & ROBSON, P. (introduced by JAMES, G.W.L.) Potentiation of anaphylactic bronchoconstriction by non-steroidal anti-inflammatory agents, 442P
MITCHELL, H.W. *see* LULICH, K.M., 71
MITCHELL, SHEILA M., POYSER, N.L. & WILSON, N.H. Effect of *p*-bromophenacyl bromide, an inhibitor of phospholipase $A_2$ on prostaglandin production by the guinea-pig uterus, 295P
MITCHELSON, F. *see* CLARK, A.L., 323
MIYAMOTO, MARIA E. *see* ABOULAFIA, JEANNINE, 223
MOBAROK ALI, A.T.M. *see* FOZARD, J.R., 276P
MOBAROK, ALI, A.T.M. *see* FOZARD, J.R., 416P
MOIR, D.C. *see* WEIR, R.D., 317P
MONCADA, S. *see* BUNTING, S., 296P
MOULIN, M.A. *see* CHICHMANIAN, R.M., 475P
MOUNIE, J. *see* DUBOST, M., 436P
MOURIES, M.A. *see* DUMEUR, G., 437P
MUID, R. *see* DALE, MAUREEN M., 288P
MUIR, T.C. *see* CORBETT, A.D., 281P
MYERS, R.D. *see* METCALF, G., 489
MYLERCHARANE, E.J. *see* DUCKWORTH, J.W., 444P

N

NAHAS, G.G. *see* DESOIZE, B., 419P
NAHORSKI, S.R. & SMITH, BRENDA M. Characteristics and altered sensitivity of cerebral $\beta$-adrenoceptors assessed by [$^3$H]-propranolol binding, 435P
NARGEOT, J. *see* GARNIER, D., 410P
NASJLETTI, A. *see* COLINA-CHOURIO, J., 165
NEAL, M.J. *see* FILE, SANDRA E., 465P
NELSON, D.J. *see* COTTNEY, J., 311P
NICOL, C.J.M. *see* BLAKELEY, A.G.H., 294P
NICHOLLS, P.J. *see* LUSCOMBE, D.K., 312P
NICHOLSON, A.N. *see* CLARKE, CORAL H., 439P
NICOLAOU, N.M. *see* ABUTHNOTT, G.W., 272P
NIJKAMP, F.P. *see* DE JONG, W., 593
NIKOLAOU, N.M. *see* BARLOW, R.B., 613
NILSSON, G. *see* LUNDELL, L., 17
NISTRI, A. *see* CONSTANTI, A., 583

O

OBERLANDER, C. *see* BOISSIER, J.R., 452P
OLLAGNIER, M. *see* FARGES, J.P., 409P
OSBORNE, D.J. *see* BOOT, J.R., 471P
OVERMAN, H.J. *see* GORDON, J.L., 300P
OWEN, F. *see* BOURNE, R.C., 291P

P

PAIVA, A.C.M. *see* ABOULAFIA, JEANNINE, 223
PAIVA, THEREZINHA B. *see* ABOULAFIA, JEANNINE, 223
PAPADIMITRIOU, A. *see* CHEVILLOTTE, E., 433P
PARK, W.K. (deceased) *see* REGOLI, D., 304P
PARRATT, J.R. & STURGESS, R.M. Effect of a new anti-inflammatory drug, flurbiprofen, on respiratory, haemodynamic and metabolic responses to *E. coli* endotoxin shock in cat, 547
PARRATT, J.R. *see* MARSHALL, R.J., 407P
PARROTT, D.P. *see* LEWIS, D.A., 441P
PARSONS, M.E. *see* BUNCE, K.T., 149
PARVEZ, H. *see* ISMAHAN, G., 450P
PARVEZ, S. *see* ISMAHAN, G., 450P
PASCAUD, X.B. & ROGER, A.R. Is the gastric antisecretory property of clonidine in rats of central origin?, 419P
PAUBLANT, D. *see* ADOLPHE, M., 448P
PEARSON, J.D. *see* DE BONO, D.P., 466P
PECK, M.J. *see* FIRRELL, J.C., 310P
PEPPER, C.M. *see* HILL, R.G., 459P
PERSSON, C.G.A. Inhibitory innervation of cat sphincter of Oddi, 477
PETERFALVI, M. *see* BOISSIER, J.R., 452P
PHILLIPS, S.W. *see* GARDNER, C.R., 267P
PILOT, MARIE-ANNE (introduced by D'MELLO, A.). Indomethacin and acid secretion in isolated perfused canine stomach, 470P
PLOTKINE, M. *see* BOULU, R.G., 417P
PLUMMER, N.A. *see* BLACK, A. KOBZA, 317P
POLLOCK, D. *see* GIBSON, A., 302P
POOL, M. *see* FILE, SANDRA E., 465P
PORQUET, D. *see* COHEN, Y., 435P
POURRIAS, B. *see* GARNIER, D., 410P
POYSER, N.L. *see* MITCHELL, SHEILA M., 295P
PUECH, R. *see* ALRIC, R., 450P
PYCOCK, C.J. *see* JENNER, P.G., 469P

Q

QUERMONNE, M.A. *see* MARCY, R., 437P

R

RAINE, A.E.G. *see* CHUBB, I.W., 430P
RAITERI, M. *see* BAKER, G.B., 420P
RAJADHYAKSHA, V. *see* EAKINS, K.E., 333
RAPER, C. *see* DUCKWORTH, J.W., 444P
RAUTHIER, J.A. *see* CHARLIER, R.H., 411P
RAVAZZANI, C. *see* BRIATICO, G., 173
RAZA-BUKHARI, A. *see* ISMAHAN, G., 450P
REDFERN, P.H. *see* BROWN, F., 561
REDFERN, P.H. *see* DAWES, P., 464P
REGOLI, D., ST-LOUIS, J. & PARK, W.K. (deceased). Factors regulating the time-course of the relaxation of rabbit aorta strips after contraction by angiotensin II, 304P
REIDERER, P. *see* AMBROZI, L., 423P
REITER, M. *see* HONERJAGER, P., 415P
REYNOLDSON, J.A. *see* CHEN, C-S., 517
RIBES, G. *see* LOUBATIERES, A.L., 429P
RICHARD, J.C. *see* CHARLIER, R.H., 411P
RICHARDS, C.D. & SMAJE, J.C. Anaesthetics depress sensitivity of cortical neurones to L-glutamate, 347
RICHARDS, I.M. *see* JACKSON, D.M., 201P
RICHARDS, J.G. Autoradiographic evidence for the effects of specific uptake inhibitors on selective accumulation of $[^3H]$-5-HT by supra-ependymal nerve terminals and for localization of binding sites for $[^3H]$-DLSD, 424P
RICHARDSON, P.D.I. The actions of natural secretin on the small intestinal vasculature of the anaesthetized cat, 127
RICHER, G. *see* DUPUIS, B., 409P
RILEY, G.J. *see* BOURNE, R.C., 291P
RITCHIE, ISOBEL M. *see* BUDNY, J., 3
ROACH, A.G. *see* DAY, M.D., 505
ROBERTS, FIONA & STEPHENSON, R.P. The kinetics of competitive antagonists on guinea-pig ileum, 57
ROBERTS, P.J. *see* HILLIER, K., 426P
ROBSON, P. *see* MILLER, P., 442P
ROGER, A.B. *see* PASCAUD, X.B., 419P
ROLLINGS, NITA A. *see* BUNCE, K.T., 149
ŘMER, D. *see* HILL, R.C., 270P
ROSENGREN, ELSA *see* *see* HENNINGSSON, S., 401
ROSS, S.B. Long-term effects of N-2-chloroethyl-N-ethyl-2-bromobenzylamine hydrochloride on noradrenergic neurones in rat brain and heart, 521
ROUGIER, O. *see* GARNIER, D., 410P
ROUSSELLE, J.C. *see* FILLION, G., 425P.
ROSSIGNOL, P. *see* BOULU, R.G., 417P
ROUZAIRE-DUBOIS, B. *see* CHEVILLOTTE, E., 433P
ROWLAND, M. *see* BENDING, M.R., 472P

S

SAEED, S.A. *see* COLLIER, H.O.J., 193
SAEED, S.A. *see* McDONALD-GIBSON, WENDY J., 573
SANGER, G.J. & WATT, A.J. A postsynaptic action of prostaglandin $E_1$ on sympathetic responses in guinea-pig ileum, 290P
SAVINI, E.C. *see* CHICHMANIAN, R.M., 475P
SCHMIDT, M. *see* IMBS, J.L., 413P
SCHMITT, H. *see* DELBARRE, B., 443P
SCHNEIDER, C. *see* COLLIER, H.O.J., 9
SCHNEIDER, C. *see* McDONALD-GIBSON, WENDY J., 573
SCHROER, R. *see* EAKINS, K.E., 333
SCHWATZ, J. *see* IMBS, J.L., 413P
SCOFIELD, P. *see* CONOLLY, M.E., 448P
SEGAL, M. Glutamate antagonists in rat hippocampus, 341
SENIOR, JUDITH *see* FOY, J.M., 308P
SENIOR, JUDITH *see* FOY, J.M., 316P
SENON, DANIELLE *see* DELBARRE, B., 443P
SERRE, M. *see* DUPUIS, B., 409P
SILERMAN, A.P. Behavioural changes in rats suggesting drug-induced headache, 439P
SMAJE, J.C. *see* RICHARDS, C.D., 347
SMAJE, J.C. General anaesthetics and the acetylcholine-sensitivity of cortical neurones, 359
SMAJE, L.H. *see* FERREIRA, S.H., 201
SMALL, R.C.COX, B., 315P
SMITH, BRENDA M. *see* NAHORSKI, S.R., 435P
SMITH, G.W. & SOMOVA, LILIANA I. Renal function and renal venous prostaglandin concentrations during different stages of experimental renal hypertension in the rat, 253
SMITH, J.W. *see* EVANS, R.H., 109
SMITH, M.J.H. *see* BOLAM, J.P., 472P
SMITH, L.A. *see* KRUK, Z.L., 468P
SMYTH, D.G. *see* BIRDSALL, N.J.M., 460P
SNELL, C.R. *see* BIRDSALL, N.J.M., 460P

SOH, K.S. *see* BARLOW, R.B., 613
SOMOVA, LILIANA I. *see* SMITH, G.W., 253
SOUBRIE, P. *see* BOISSIER, J.R., 454P
SPARROW, M.P. *see* LULICH, K.M., 71
SPENCE, J.C. *see* GIBSON, A., 302P
STEINESS, EVA & VALENTIN, N. Myocardial digoxin uptake: dissociation been digitalis-induced inotropism and myocardial loss of potassium, 183
STEINESS, EVA & VALENTIN, N. Myocardial uptake of digoxin in chronically digitalized dog, 553
STEPHENSON, R.P. *see* ROBERTS, FIONA, 57
STEVENSON, I.H. *see* MARSHALL, P.B., 309P
ST-LOUIS, J. *see* REGOLI, D., 304P
STOCLET, J.C. *see* LUGNIER, C., 434P
STOCKHAM, M.A. *see* DAVEY, A., 289P
STONE, BARBARA M. *see* CLARKE, CORAL H., 439P
STURGESS, R.M. *see* PARRATT, J.R., 547
SURGUE, M.F. *see* CARLTON, K.G., 271P
SUMMERS, R.J. & TILLMAN, JANET. The effects of AH 5158 on metabolism of [$^3$H]-(−)noradrenaline released from the cat spleen by nerve stimulation, 280P
SUTAK, M. *see* JHAMANDAS, K., 101
SVENSSON, S.E. *see* LUNDELL, L., 17
SZABADI, E. *see* BEVAN, P., 239
SZABADI, E. *see* BEVAN, P., 418P

T

TAYLOR, M. Effects of L-tryptophan and L-methionine on activity in the rat, 117
TAYOR, S.H. & THADANI, U. Oxprenolol in angina pectoris, 412P
TELLA, A. Studies on *Azadiracta indica* in malaria, 318P
TEMPLETON, D. *see* CORBETT, A.D., 281P
TENENHOUSE, A. *see* FAST' D., 605
THADANI, U. *see* TAYLOR, S.H., 412P
THEEBOT, M.H. *see* BOISSIER, J.R., 454P
TILLMAN, JANET *see* SUMMERS, R.J., 280P
TILMISANY, A.K. *see* GILLESPIE, J.S., 47
TODA, N. Influence of dopamine and noradrenaline on isolated cerebral arteries of the dog, 121
TONGE, SALLY R. *see* FLUDDER, JOAN M., 422P
TREMBLAY, D. *see* DUMEUR, G., 437P
TRENDELENBERG, U. *see* HONERJAGER, P., 415P
TRUCHOT, R. *see* DUBOST, M., 436P
TULLOCH, I.F. *see* ARBUTHNOTT, G.W., 272P
TURNBULL, M.J. *see* EFELLAH, M.S., 274P
TURNBULL, M.J. & WATKINS, J.W. Determination of halothane-induced sleeping time in the rat: effect of prior administration of centrally active drugs, 27
TURNBULL, M.J. & WATKINS, J.W. Change in sensitivity to halothane in the rat following administration of sodium barbitone in the drinking water, 285P
TURNER, P. *see* BARBHAIYA, R.H., 473P
TURNER, P. *see* KIRBY, MARILYN J., 286P

V

VALENTIN, N. *see* STEINESS, EVA, 183
VALENTIN, N. *see* STEINESS, EVA, 553
VALETTE, G. *see* LOUBATIERES, A.L., 429P
VANE, J.R. *see* BUNTING, S., 296P
VARGAFTIG, B.B. Effects of prostaglandin synthetase inhibitors and of other drugs on aggregation of plasma-free rabbit platelets by carrageenin and by thrombin, 447P
VAUGHAN WILLIAMS, E.M. *see* CHUBB, I.W., 430P
VERITY, ANNE *see* FARRAR, D.G., 431P
VINE, PAULINE, *see* DALE, M. MAUREEN, 229

W

WADDINGTON, J.L. (introduced by CROW, T.J.) Behavioural model of GABA-facilitating action of benzodiazepines: rotational behaviour after unilateral intranigral injection of chlordiazepoxide, 453P
WALKER, J.M.G. *see* CARDINAL, D.C., 463P
WALKER, J.R. *see* BOLAM, J.P., 472P
WALTER, D.S. *see* COWAN, A., 275P
WATKINS, J.C. *see* BISCOE, T.J., 373
WATKINS, J.W. *see* TURNBULL, M.J., 27
WATKINS, J.W. *see* TURNBULL, M.J., 285P
WATT, A.J. *see* SANGER, G.J., 290P
WEBB, SANDRA N. *see* LEWIS, D.M., 467P
WEBSTER, R.A. *see* FAGG, G.E., 440P
WEIL-FUGAZZA, J. *see* BOISSIER, J.R., 454P
WEIR, R.D., MOIR, D.C., ERSKINE, Z.G., GALLON, S.C., DAVIDSON, J.F., CROOKS, J. & CHRISTOPHER, L.J. Aberdeen-Dundee medicines evaluation and monitoring group, 317P
WEPIERRE, J. *see* COHEN, Y., 435P
WESTLING, H, *see* HÖGLUND, ELIN, 43
WESTON, A.H. *see* JETLEY, M., 287P
WESTWICK, J. (introduced by LEWIS, G.P.) The effect of pulmonary metabolites of prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ on ADP-induced aggregation of human and rabbit platelets, 297P
WHEATLEY, P.L. *see* BAXTER, M.G., 269P
WHITE, T. *see* HÖGLUND, ELIN, 43
WHITTLE, B.J.R. Calcium and inhibition of histamine release from rat peritoneal mast cells by non-steroid anti-inflammatory agents, 446P
WILLIAMS, T.J. *see* FIRRELL, J.C., 310P
WILLOUGHBY, D.A. *see* ADOLPHE, M., 448P
WILSON, N.H. *see* MITCHELL, SHEILA M., 295P
WONG, P.Y.D. & YEUNG, C.H. Inhibition by amiloride of sodium-dependent fluid reabsorption in rat isolated caudal epididymis, 529
WOODS, H.F *see* BLAU, K., 474P
WOOLLARD, P.M. *see* HILLIER, K., 426P
WORCEL, M. *see* ALTMAN, J., 432P
WORCEL, M. *see* CHEVILLOTTE, E., 433P
WRIGHT, A.K. *see* ARBUTHNOTT, G.W., 272P
WRIGHT, I.G. *see* BOREHAM, P.F.L., 137

Y

YAMADA, S. *see* BROWN, D.A., 157
YEUNG, C.H. *see* WONG, P.Y.D., 529
YOSHIMURA, T. *see* AZUMA, H., 483
YOUDIM, M.B.H. *see* AMBROZI, L., 423P
YOUDIM, M.B.H. *see* BLAU, K., 474P

Z

ZAR, M.A. *see* IDOWU, O.A., 278P
ZARRINDAST, M.R. *see* KRUK, Z.L. 272P
ZARRINDAST, M.R. *see* KRUK, Z.L., 367
ZARRINDAST, M.R. *see* KRUK, Z.L., 468P

# SUBJECT INDEX

A

**Acetic acid**, effect of, reduced by propyl gallate in rodents, 573

**Acetylation**, intersubject variability of sulphadimidine, in student volunteers, 312P

**Acetylcholine**, contractual response of denervated mouse diaphragm muscle to, 109

—, dose-response curves for guinea-pig innervated and denervated cremaster muscle, 229

—, effect of indomethacin and prostaglandin on smooth muscle contracting activity of, 223

—, effect of metiamide on acid secretion stimulated by, in isolated whole rat stomach, 149

—, effects of, on cat lung strip preparation, 71

—, effect of, on guinea-pig cremaster muscle preparation, 288P

—, effect of, on guinea-pig urinary bladder, 37

—, increases incidence of ethanol withdrawal head twitches in mice, 9

—, release of, from cerebral cortex and morphine-naloxone interaction, 101

—, effect of, reduced by propyl gallate in rodents, 573

—, effect of, on inhibitory innervation of cat sphincter of Oddi, 477

—, sensitivity of cortical neurones to, effect of general anaesthetics on, 359

—, turnover of, effect of glucocorticoids on, in isolated guinea-pig ileum, 475P

—, effect of, on effect of gallamine on muscarinic receptors, 323

—, analysis of end-plate current fluctuations produced by, in rat brain, 428P

**Acetylcholine receptors**, comparison of affinity constants for muscarine-sensitive, in guinea-pig atrial pacemaker cells and ileum, 613

**Acetylmonoethylcholine**, analysis of end-plate fluctuations produced by, in rat brain, 428P

**Acid secretion**, and indomethacin, in isolated perfused canine stomach, 470P

**Action potential**, cardiac, activity-dependent prolongation of, by batrachotoxin, 415P

**$\alpha_2$-Acute phase globulin**, effect of cortisol on, in arthritic rat, 441P

**Adenine**, active transport of, and adenosine by blood platelets and cultured endothelial cells, 466P

**Adenosine**, active transport of, and adenine, by blood platelets and cultured endothelial cells, 466P

**Adenosine diphosphate**, effect of, on inhibitory innervation of cat sphincter of Oddi, 477

**Adenylate cyclase**, effect of chronic pretreatment with sympathomimetic bronchodilators on responsiveness of guinea-pig lung, *in vitro*, 274P

**Adipose tissue**, glucose utilization in, of man and rat, inhibition of, by catecholamine-induced anorectic drugs, 436P

**Adrenaline**, coronary vascular response to, 89

—, centrally induced hypotension and bradycardia after administration of, into area of rat nucleus tractus solitarii, 593

—, effect of indomethacin on responses of rabbit smooth muscle to, 223

—, effects of, on cat lung strip preparation, 71

—, effect of, on guinea-pig urinary bladder, 37

—, effect of subcutaneous injections of, on platelet MAO activity, 291P

—, response of denervated cremaster muscle preparation to, 229

—, in isotonic contractions of mammalian skeletal muscle, 467P

**Adrenergic nerve terminals**, further evidence that prostaglandins inhibit release of noradrenaline from, by restriction of availability of calcium, 599

**Adrenergic neurone blocking agents**, proposed site of action of, 497

**Adrenoceptor agonists**, neuronal responses to, in cerebral cortex, evidence for excitatory $\alpha$-adrenoceptors and inhibitory $\beta$-adrenoceptors, 418P

**Adrenoceptors**, of the guinea-pig urinary bladder, 37

—, presence of an excitatory, in guinea-pig urinary bladder, 37

—, excitatory, evidence for, 418P

**$\alpha$- and $\beta$-Adrenoceptors**, effects of AH5158 on, 412P

**$\alpha$-Adrenoceptors**, inhibitory, evidence for, 418P

—, cerebral, characteristics and altered sensitivity of, assessed by [$^3$H]-propranolol binding, 435P

—, presence of an inhibitory, in guinea-pig urinary bladder, 37

**$\beta$-Adrenoceptor agonists**, comparison of, autacoids and anaphylactic challenge on cat lung strip and trachea, 71

**$\beta$-Adrenoceptor antagonists**, studies with, and inhibition of milk-ejection reflex, 464P

**$\beta$-Adrenoceptor-blocking agents**, effect of, on cardiovascular effects of dopamine and $\alpha$-methyldopamine in conscious cat, 505

**Affinity constants**, comparison of, for muscarinic-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells and in ileum, 613

**AH 5158**, effects of, on metabolism of [$^3$H]-(−)noradrenaline released from cat spleen by nerve stimulation, 280P

—, site of uptake inhibition by, is probably neuronal, 280P

—, some complementary data on, an inhibitor of both $\alpha$- and $\beta$-adrenoceptors, 412P

**$\alpha$-Allokainate**, effect of, on rat and frog spinal neurones, 373

**Alphaxalone**, effect of, on sensitivity of cortical neurones to L-glutamate, 347

—, effect of, on sensitivity of cortical neurones to acetylcholine, 359

**Alprenolol**, effect of, on cardiovascular effects of dopamine in conscious cat, 505

**Amantadine**, studies on mechanism of action of, 561

—, effect of, on binding and efflux characteristics of noradrenaline and guanethidine, 497

**Amfepramone,** activity of, on two models of obesity in animals, 437P

**Amiloride,** inhibition by, of sodium-dependent fluid reabsorption in rat isolated caudal epididymis, 529

**Amine neurotransmitters,** putative, effect of *p*-chloro-*β*-phenylethylamine on uptake and release of, in rat brain, 420P

**Amines,** accumulation of biogenic, by rabbit erythrocytes, 294P

**Amino acids,** responses evoked by, recorded from lobster muscle fibre and frog spinal cord, antagonism of, by some antihistamines, 583

—, acidic, rat hippocampal cellular responses to, 341

—, aromatic, in plasma, brain and other tissues, effect of aminophylline on, 533

—, excitatory, structure-activity relations of, on frog and rat spinal neurones, 373

—, endogenous, release of, from cat spinal cord *in vivo*, 440P

DL-**2-amino-4-thiosulphonylbutyric acid,** effect of, on rat and frog spinal neurones, 373

L-**2-amino-3-thiosulphonylpropionic acid,** effect of, on rat and frog spinal neurones, 373

*γ*-**Aminobutyric acid,** responses evoked by, from lobster muscle fibre and frog spinal cord, effect of antihistamines on, 583

—, levels of, in substantia nigra, ethanolamine-O-sulphate-induced changes in, behavioural effects of, 454P

—, -facilitating action of benzodiazepines; rotational behaviour after unilateral intranigral injection of chlordiazepoxide, a behavioural model of, 453P

—, metabolism in mouse, effect of nandrolone on, 401

—, release of, from isolated synaptosomal fractions from mouse forebrain, 569

—, antagonists of, reversal of action of, by barbiturates, 456P

—, evidence for naloxone and opiates as, 458P

**Aminophylline,** effects of, on cat lung strip preparation, 71

—, effect of, on response of dog cerebral arteries to dopamine and noradrenaline, 121

—, effect of, on aromatic amino acids in plasma, brain and other tissues and on brain 5-HT metabolism, 533

**Amiodarone,** -like, haemodynamic profile, an indolizine with an, 411P

**Amphetamine,** decreases incidence of ethanol withdrawal head twitches in mice, 9

—, activity of, on two models of obesity in animals, 437P

—, -type, anorectic drugs, peripheral effects of, inhibition of catecholamine-induced lipolysis, respiration, glucose utilization in adipose tissue of man and rat, 436P

(+)-**Amphetamine,** effect of, on binding and efflux characteristics of noradrenaline and guanethidine, 497

—, effects of, modification of, by oestrogen, on noradrenaline metabolism in discrete areas of brain, 422P

—, effect of, on uptake and release of brain monoamines, 272P

**Ampicillin,** fluorimetric determination of, 473P

**Amylobarbitone,** effect of pretreatment with, on halothane-induced sleeping time in rats, 27

**Anaesthesia,** blood pressure and respiration recording in the anaesthetized cat, 316P

—, duration of halothane-induced, following administration of sodium barbitone in drinking water to the rat, 285P

**Anaesthetics,** depress sensitivity of cortical neurones to L-glutamate, 347

—, general, and the acetylcholine sensitivity of cortical neurones, 359

**Analgesia,** produced by morphine when acting from the liquor space, 383

**Anaphylactic bronchoconstriction,** potentiation of, by non-steroidal anti-inflammatory agents, 442P

**Anaphylaxis,** action of metiamide in, *in vivo* in the guinea-pig, 303P

—, passive cutaneous, effect of N-5′ on, 483

**Angina pectoris,** oxprenolol in, 412P

**Angiotensin,** effect of indomethacin and prostaglandin on the smooth muscle contracting activity of, and other agonists, 223

—, molecular mechanism of post-nephrectomy uterine supersensitivity to, 433P

**Angiotensin II,** *in vitro,* vascoconstrictor supersensitivity to, prostaglandin $E_2$ released from kidneys of genetic hypertensive rats contributes to, 462P

—, regulation of time course of relaxation of rabbit aorta strips after contraction by, 304P

**Anococcygeus muscle,** action of tetraethylammonium chloride on response of, to motor and inhibitory nerve stimulation and to some drugs, 47

—, effect of corticosteroids on responses of, to nerve stimulation in the pithed rat, 302P

—, potassium-induced relaxations of the rat, 305P

**Anorectic drugs,** act through either dopaminergic or 5-HT mechanisms in the brain, 272P

—, amphetamine-type, peripheral effects of, 436P

—, activity of, on two models of obesity in animals, 437P

**Anorectics,** effects of, on uptake and release of brain monoamines, 272P

—, antagonism of responses to, by selective receptor blockers, 468P

**Anorexia,** induced by mazindol, is mediated by activation of dopaminergic mechanisms, 367

**Anoxia,** coronary vascular response to, 89

**Antazoline,** kinetics of, in guinea-pig ileum, 57

—, effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583

**Antihypertensive drugs,** studies on the efficacy of indapamide, a new, in cats and rats, 282P

**Anti-inflammatory activity,** sponge implantation test in rat as a model for screening, 472P

**Anti-inflammatory agents,** non-steroid, inhibition by, of release of histamine from rat peritoneal mast cells, 446P

—, non-steroidal, potentiation of anaphylactic bronchoconstriction by, 442P

**Anti-inflammatory drug,** new, flurbiprofen, effect of, on respiratory, haemodynamic and metabolic responses to *E. coli* endotoxin shock in cat, 547

**Anti-inflammatory effect,** topical, of propyl gallate in rodents, 573

**Antiarrythmic drugs,** use of a method for experimental study of arrythmias following myocardial infarction in closed-chest dog, to test, 409P

**Antihistamines,** antagonism by some, of amino acid-evoked responses recorded from lobster muscle fibre and frog spinal cord, 583

**Antinociceptive effect,** local, of propyl gallate in rodents, 573

**Aorta,** factors regulating time course of relaxation of strips of rabbit, after contraction by angiotensin II, 304P

**Apomorphine,** decreases incidence of ethanol withdrawal head twitches in mice, 9
**Aprotinin,** pretreatment with, prevents hypotension in response to experimental trypanosomiasis in rabbits, 137
—, reduces prostaglandin releasing action of kininogen in rabbit perfused kidney, 165
**Arachidonic acid,** addition of exogenous, cannot prevent inhibition of PG synthesis by guinea-pig uterus in presence of *p*-bromophenacyl bromide, 295P
—, effects of aspirin and indomethacin on 5-HT release induced by, in rat platelets, 299P
—, paw oedema induced by, in the rat, 311P
—, effect of, reduced by propyl gallate in rodents, 573
**Arrythmias,** experimental study of, following myocardial infarction in closed-chest dog, its use as a test for anti-arrythmic drugs, 409P
**Arthritic rat,** effect of cortisol on $\alpha_1$-macroglogulin and $\alpha_2$-acute phase globulin in, 441P
**Asparagine,** effect of, on rat and frog spinal neurones, 373
**Aspartate,** responses evoked by, from lobster muscle fibre and frog spinal cord, effect of antihistamines on, 583
—, effect of, on rat and frog spinal neurones, 373
—, effect of, on rat hippocampal cells, 341
**Aspirin,** class experiment with student volunteers illustrating influence of formulation on absorption of, 312P
—, effects of, on extent and duration of malondialdehyde production by rat platelets, 299P
—, effects of, on 5-HT release induced by arachidonic acid and collagen in rat platelets, 299P
—, effectiveness of, against inflammation, 573
**Atenolol,** regional myocardial blood flow and S-T segment in canine ischaemic myocardium, 411P
**ATP,** effect of, on inhibitory innervation of cat sphincter of Oddi, 477
—, decreases incidence of ethanol withdrawal head twitches in mice, 9
**(Na-K)-ATPase,** prepared from human heart, role of potassium in inhibition by cardiac glycosides of, 413P
**Atrial fibres,** frog, effects of tryptamine on mechanical and electrical activities of sino-atrial frog auricular trabeculae, 410P
**Atropine,** affinity constant for, in guinea-pig innervated and denervated cremaster muscle, 229
—, effect of, on fenfluramine-induced glucose uptake into isolated rat hemidiaphragm, 286P
—, effect of, on response of dog cerebral arteries to dopamine and noradrenaline, 121
—, kinetics of, in guinea-pig ileum, 57
—, salivation and vasodilatation in the dog following administration of, 201
—, effect of, on inhibitory innervation of cat sphincter of Oddi, 477
—, effect of, on effect of gallamine on muscarinic receptors, 323
—, effect of, on effect of general anaesthetics on sensitivity of cortical neurones to acetylcholine, 359
—, effect of, on cardiovascular effects of $\alpha$-methylnoradrenaline after administration into area of rat nucleus tractus solitarii, 593
**Attention,** -sustaining capacity model for psychopharmacological research, 437P
**Auricular trabeculae,** frog, effects of tryptamine on mechanical and electrical activities of, 410P
**Autonomous control,** of heart, myocardial blood flow and S-T segment in canine ischaemic myocardium, 410P
**Autonomic drugs,** and the eye, 315P
***Azadiracta indica,*** studies on, in malaria, 318P

B

**Barbiturates,** reversal of action of GABA antagonists by, 456P
**Batrachotoxin,** activity dependent prolongation of cardiac action potential and positive inotropic effect, 415P
**Behaviour,** circling, a comparison of, induced in nigrostriatal lesioned rats after peripheral administration of indole derivatives, 452P
—, rotational, after intranigral injection of chlordiazepoxide, 453P
—, in chickens, effects of activation of $H_1$- and $H_2$-receptors on, 443P
—, rat social, effect of *p*-chlorophenylalanine and $\alpha$-methyltryptophan on, 455P
—, dopamine-dependent, in rodents, interaction of clonidine with, 469P
**Behavioural changes,** in rats, suggesting drug-induced headache, 439P
**Behavioural effects,** of EOS-induced changes in substantia nigra GABA levels, 454P
**Benziloyltropine methyllodide,** kinetics of, in guinea-pig ileum, 57
**Benzodiazepines,** GABA-facilitating action of, rotational behaviour after unilateral intranigral injection of chlordiazepoxide, 453P
**1,5-Benzodiazepines,** effect of, on sleep of man, 439P
**Benzylamine,** effect of, as substrate on monoamine oxidase activity in rat brain and spinal cord, 279P
**Bethanidine,** effect of, on cardiovascular effects of dopamine in conscious cat, 505
**Bicuculline,** effect of, on firing rate of cells in the nucleus accumbens and preoptic area in the rat, 267P
**Blood flow,** myocardial, autonomous control of, in canine ischaemic myocardium, 410P
—, regional myocardial, atenolol and S-T segment in canine ischaemic myocardium, 411P
—, effect of chloracyzine on coronary, 289
**Blood pressure,** and respiration of the anaesthetized cat, 316P
—, systemic arterial, effect of methylnoradrenaline, noradrenaline and adrenaline on, after administration into area of rat nucleus tractus solitarii, 593
—, effect of dopamine on, in conscious cat, 505
—, effect of inapamide on, in rats and cats, 282P
**Blood-brain barrier,** long-lasting effects of opening of, on modifications induced by intracarotid injection of noradrenaline, 417P
**Bradycardia,** centrally induced, after administration of $\alpha$-methylnoradrenaline into area of nucleus tractus solitarii of rat, 593
**Bradykinin,** and functional vasodilatation in the salivary gland, 201
—, effect of indomethacin and prostaglandin on smooth muscle contracting activity of, 223
—, effect of, on renal prostaglandin release in rabbit perfused kidney, 165
**Brain stem,** rat, centrally induced hypotension and bradycardia after administration of $\alpha$-methylnoradrenaline into area of nucleus tractus solitarii of, 593
**British Pharmacological Society,** Proceedings of, University of Oxford, September 15–17, 1976, 407P–475P

—, Proceedings of, Dundee, July 15–16, 1976, 267P–318P

*p*-**Bromophenacyl bromide**, effect of, on prostaglandin production by guinea-pig uterus, 295P

**Bronchoconstriction**, anaphylactic, potentiation of, by non-steroidal anti-inflammatory agents, 442P

—, effect of sodium cromoglycate on reflex, in the dog, 301P

**Bronchodilators**, circulatory effects of oral and subcutaneous ibuterol, a new, in normal subjects, 43

—, effect of chronic pretreatment with, on responsiveness of guinea-pig lung adenylate cyclase *in vitro*, 274P

**Bufexamac**, therapeutic value of, applied after u.v. irradiation, 573

**Buprenorphine**, effect of, on HVA, 5-HIAA and MHPG concentrations in rat forebrain, 275P

**Burimamide**, effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583

C

**C-Fragment**, of $\beta$-lipotropin, peptides derived from, interaction of, with brain opiate receptors, 460P

**C-Terminal octapeptide of cholecystokinin**, effect of, on inhibitory innervation of cat sphincter of Oddi, 477

**Caffeine**, effect of, on aromatic amine acids in plasma, brain and other tissues, and on brain 5-HT metabolism, 533

**Calcium**, and inhibition and histamine release from rat peritoneal mast cells by non-steroid anti-inflammatory agents, 446P

—, availability of, restriction of, further evidence that prostaglandins inhibit release of noradrenaline from adrenergic nerve terminals by, 599

—, effect of, on mechanism by which dibutyryl cyclic AMP potentiates secretory effect of carbachol on rat exocrine pancreas *in vitro*, 605

—, effect of, on release of GABA from isolated synaptosomal fractions from mouse forebrain, 569

—, effect of catecholamines on influx of, and development of tension in denervated mouse diaphragm muscle, 109

—, effect of part played by, in contraction by angiotensin II on time course of relaxation of rabbit aorta strips, 304P

**Calcium ions**, hypothermia induced by, in unanaesthetized cat, 489

**Carbachol**, effect of, on protein secretion from rat exocrine pancreas *in vitro*, effect of dibutyryl cyclic AMP on, 605

—, effect of ketamine on motor responses to, in rat anococcygeus muscle, 247

—, effect of, on effect of gallamine on muscarinic receptors, 323

—, effect of TEA on response of rat anococcygeus muscle to, 47

—, increases incidence of ethanol withdrawal head twitches in mice, 9

**Carbohydrate metabolism**, and MAO activity, 450P

**Carbon tetrachloride**, action of, and the products of metabolism in sheep on *Fasciola hepatica*, 395

**Carbonic anhydrase inhibitors**, effects of, on cerebral cortex oxygen availability and resistance to hypoxia, 417P

**Cardiovascular effects**, of dopamine after central administration into conscious cat, 505

—, of $\alpha$-methylnoradrenaline, after administration into area of rat nucleus tractis solitarii, 593

**Cardiovascular structures**, central, in cats, effects of activation of $H_1$- and $H_2$-receptors on, 443P

**Carrageenin**, aggregation of plasma free rabbit platelets by, effects of prostaglandin synthetase inhibitors on, 447P

**Catecholamine**, prostaglandin synthesis stimulated by, in rat brain synaptosomes, 426P

—, -induced lipolysis, inhibition of, by amphetamine-type anorectic drugs, 436P

**Catecholamines**, effects of, on cat lung strip preparation, 71

—, metabolism of, effect of amantadine on, 561

—, effect of, on influx of calcium and development of tension in denervated mouse diaphragm muscle, 109

—, receptors involved in growth and secretion mediated by, in rat parotid gland, 281P

**Caudal epididymis**, rat isolated, effect of amiloride on sodium-dependent fluid reabsorption in, 529

**Cells**, rat hippocampal pyramidal, effect of, glutamate antagonists on, 341

—, cultured endothelial, active transport of adenine and adenosine by, 466P

—, rat peritoneal mast, release of histamine from, inhibition of, by non-steroid anti-inflammatory agents, 446P

—, guinea-pig atrial pacemaker, comparison of affinity constants for muscarine-sensitive acetylcholine receptors in, 613

**Cephalexin**, fluorimetric determination of, 473P

**Cerebral arteries**, influence of dopamine and noradrenaline on isolated, of the dog, 121

**Cerebral cortex**, neuronal responses to adrenoceptor agonists in, evidence for excitatory $\alpha$-adrenoceptors and inhibitory $\beta$-adrenoceptors, 418P

**Chloracyzine**, decrease of myocardial oxygen consumption induced by, 189

*p*-**Chloroamphetamine**, decreases incidence of ethanol withdrawal head twitches in mice, 9

—, effect of, on uptake and release of brain monoamines, 272P

**Chlordiazepoxide**, effect of, on suppressed responding, 451P

—, intranigral injection of, rotational behaviour after, 453P

—, effects of, on social interaction in rats, 465P

**Chlorimipramine**, effect of, on accumulation of 5-HT by supra-ependymal nerve terminal, 424P

**2-Chloro-10-(3-diethylaminopropionyl)phenothiazine hydrochloride**, see chloracyzine, 189

*N*-**2-chloroethyl-*N*-ethyl-2-bromobenzylamine hydrochloride**, effect of, on noradrenergic neurones in rat brain and heart, 521

*p*-**Chlorophenylalanine**, effect of, on rat social behaviour, 455P

—, decreases incidence of ethanol withdrawal head twitches in mice, 9

*p*-**Chloro-$\beta$-phenylethylamine**, effect of, on uptake and release of putative amine neurotransmitters in rat brain, 420P

**Chlorpheniramine**, effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583

**Chlorphentermine**, effect of, on uptake and release of brain monoamines, 272P

**Chlorpromazine**, comparative studies on effects of, with those of metoclopramide on EEG in conscious rat, 269P

—, effect of, on suppressed responding, 451P

**Cholecystokinin**, C-terminal octapeptide of, effect of, on inhibitory innervation of cat sphincter of Oddi, 477

**Clobazam**, effect of, on sleep of man, 439P

**Clonidine**, inhibitory effect of, on peripheral adrenergic synapse, 278P

—, gastric antisecretory property of, is it of central origin?, 419P
—, interaction of, with dopamine-dependent behaviour in rodents, 469P
**Cocaine,** comparison of effect of, with that of ketamine on motor responses of rat anococcygeus muscle, 247
—, effect of, on $K^+$-induced relaxations of the rat anococcygeus muscle, 305P
—, effect of, on responses of isolated rat vas deferens to noradrenaline and methoxamine, 280P
—, effect of, on binding and efflux characteristics of noradrenaline and guanethindine, 497
**Collagen,** effects of aspirin and indomethacin on 5-HT release induced by, in rat platelets, 299P
**Contraceptives,** effects of, on drug metabolism in various animal species, 173
**Contractile tissue,** atrial and ventricular cardiac contractile, heterogeneous reactivity of, 409P
**Corn oil,** headache induced by, behavioural changes in rats suggesting, 439P
**Coronary vasodilator metabolite,** release of, from guinea-pig isolated perfused heart stimulated by catecholamines, histamine and electrical pacing and by exposure to anoxia, 89
**Corticosteroids,** effects of, on responses of anococcygeus and gastrocnemius muscles to nerve stimulation in the pithed rat, 302P
**Corticosterone,** effect of, on neuromuscular function in the pithed rat, 302P
**Cortisol,** effect of, on $\alpha_1$-macroglobulin and $\alpha_2$-acute phase globulin in arthritic rat, 441P
**Creatinine,** effect of, on binding of *o*-methyl red to human albumin, 283P
**Cremaster muscle,** guinea-pig denervated, as a pharmacological preparation, 229
—, simple *in vitro* preparation of, showing properties of slow tonic skeletal muscle, 288P
—, a simple *in vitro* preparation of guinea-pig, 288P
**Current,** end-plate, fluctuations in, analysis of, produced by acetylcholine and acetylmonoethylcholine in rat muscle, 428P
**Cyanosis,** effect of development of, on fibrin accumulation in rabbit skin homografts, 292P
**Cyclic AMP phosphodiesterase,** evolution of aortic and cardiac, during onset of mineralocorticoid hypertension in rat, 434P
**Cyclophosphamide,** maintains level of insoluble fibrinogen in rabbit skin homografts in the absence of cyanosis, 292P
**Cyproheptadine,** effect of, on effect of N-5′, 483
—, effect of, on isolated rabbit ear artery and aorta, 211
**Cytochrome C,** action of, on transmembrane potentials of normal and hypoxic guinea-pig myocardial strips, 431P

D

**D600,** effects of, on electrical and mechanical activity in rat portal vein, 287P
**Decamethonium,** pharmacological response to labelled, in cat muscle, 141
**Delta-9-tetrahydrocannabinol,** and macromolecular synthesis, 419P
**Dependence,** comparison of self-administration of morphine and pentazocine in the rhesus monkey, 270P
**(—)-Deprenil,** in treatment of Parkinson's disease, 423P
**Desipramine,** effect of, on accumulation of 5-HT by supraependymal nerve terminals, 424P
—, effect of, on binding and efflux characteristics of noradrenaline and guanethidine, 497
—, effect of, on effect of *N*-2-chloroethyl-*N*-ethyl-2-bromobenzyl amine hydrochloride, on rat brain and heart, 521
**Diaphragm,** effect of catecholamines on calcium influx and development of tension in denervated, in the mouse, 109
**Dibutyryl cyclic adenosine-3′,5′-monophosphate,** effect of, on protein secretion from rat exocrine pancreas *in vitro*, 605
—, decreases incidence of ethanol withdrawal head twitches in mice, 9
—, effect of metiamide on acid secretion stimulated by, in isolated whole rat stomach, 149
—, increases incidence of ethanol withdrawal head twitches in mice, 9
**Diethylpropion,** effect of, on uptake and release of brain monoamines, 272P
**Digitalization,** in dog, chronic, myocardial uptake of digoxin during, 553
**Digoxin,** myocardial uptake of, in chronically digitalized dog, 553
—, myocardial uptake of, 183
**Dihydro-erythroidine,** effect of, on effect of general anaesthetics on sensitivity of cortical neurones to acetylcholine, 359
**L-3,4-Dihydroxyphenylalanine,** action of, on single cortical neurones microelectrophoretically applied, 239
—, effect of, on rat and frog spinal neurones, 373
**Diisopropylphosphorofluoridate,** effect of, on effect of gallamine on muscarinic receptors, 323
**N-(3′,4′-dimethoxycinnamoyl)anthranilic acid (N-5′),** pharmacological properties of, 483
***N,N*-Dimethyl-5-HT,** stimulates release of transmitter from rabbit cardiac sympathetic nerves, 276P
**Diphenhydramine,** effect of, on effect of N-5′, 483
—, effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583
**Dipyridamole,** effect of, on inhibitory innervation of cat sphincter of Oddi, 477
**Diquat,** *in vivo* absorption studies with, in the dog, 284P
**Disulfiram,** effect of, on cardiovascular effects of dopamine and $\alpha$-methyldopamine in conscious cat, 505
**Dobutamine,** effects of, in early stages of acute myocardial infarction in dog, 407P
**Domoate,** effect of, on rat and frog spinal neurones, 373
**DOPA,** action of, on single cortical neurones, 239
**L-DOPA,** effect of, on rat and frog spinal neurones, 373
—, in treatment of Parkinson's disease, 423P
—, decreases incidence of ethanol withdrawal head twitches in mice, 9
**Dopamine,** comparison of D and L-tryptophan on cerebral metabolism of 5-HT and, in the dog, 3
—, decreases incidence of ethanol withdrawal head twitches in mice, 9
—, effect of anorectic drugs on uptake and release of, in the brain, 272P
—, effect of mazindol on metabolism of, in rat brain, 271P
—, feed-back control of release of, 272P
—, influence of, on isolated cerebral arteries of the dog, 121
—, effect of amantadine on brain concentration of, 561
—, effect of, on effect of *N*-2-chloroethyl-*N*-ethyl-2-bromobenzyl amine hydrochloride on rat brain and heart, 521

—, cardiovascular effects of, after central administration into conscious cat, 505
—, -dependent behaviour in rodents, interaction of clonidine with, 469P
**Dopamine-β-hydroxylase,** stimulated release of, from intact organs of normo- and hypertensive rats, 275P
—, long-term effects of propranolol on, in superior cervical ganglia of rabbit, 430P
**Dopaminergic mechanisms,** mazindol anorexia is mediated by activation of, 367
**Dopaminergic modulation,** of release of histamine and SRS-A in calf, 445P
**Drug metabolism,** effects of contraceptive agents on, in various animal species, 173

E

***E. Coli* endotoxin shock in cat,** effect of a new anti-inflammatory drug, flurbiprofen, on respiratory, haemodynamic and metabolic responses to, 547
**Electroencephalogram,** comparative studies on effects of metoclopramide and known neuroleptics on, in conscious rat, 269P
**Ellagic acid,** pretreatment with, affects course of arachidonic acid-induced paw oedema in the rat, 311P
**Endotoxin shock,** *E. coli,* in cat, effect of a new anti-inflammatory drug, flurbiprofen, on respiratory, haemodynamic and metabolic responses to, 547
**Enkephalin,** actions of, on spinal cord and brain stem neurones, 458P
**Erythrocytes,** accumulation of biogenic amines by rabbit, 294P
**Ethanol,** effects of drugs affecting endogenous amines and cyclic nucleotides on withdrawal head twitches in mice, 9
—, effects of, on social interaction in rats, 465P
**Ethanolamine-*O*-sulphate,** -induced changes in substantia nigra GABA levels, behavioural effects of, 454P
**Ether,** effect of, on sensitivity of cortical neurones to L-glutamate, 347
—, effect of, on sensitivity of cortical neurones to acetylcholine, 359
**Ethylene glycol dinitrate,** headache induced by, behavioural changes in rats suggesting, 439P
**Eye,** autonomic drugs and the, 315P

F

***Fasciola hepatica,*** actions of carbon tetrachloride, hexachloroethane and the products of their metabolism in sheep on, 395
**Fatty acid,** plasma unesterified, effect of aminophylline on, 533
**Fenfluramine,** effect of, on uptake and release of brain monoamines, 272P
—, effect of some receptor antagonists on glucose uptake induced by, into isolated rat hemidiaphragm, 286P
**Fibrin,** role of lymphocytes in accumulation of, in rabbit skin homografts, 292P
**Flufenamate,** effects of, on cat lung strip preparation, 71
**Fluorimetric determination,** of ampicillin and cephalexin, 473P
**Flurbiprofen,** a new anti-inflammatory drug, effect of, on respiratory, haemodynamic and metabolic responses to *E. coli* endotoxin shock in cat, 547
**French Pharmacological Society,** Proceedings of, University of Oxford, September 15–17, 1976, 407P–475P
**Frusemide,** effect of (±)-propranolol on recovery of urinary concentration process after, in rat, 413P

G

**Gallamine,** effect of, on effect of general anaesthetics on sensitivity of cortical neurones to acetylcholine, 359
—, inhibitory effect of, on muscarinic receptors, 323
**Ganglia,** rabbit superior cervical, long-term effects of propranolol on tyrosine hydroxylase and dopamine-β-hydroxylase in, 430P
**Gastric secretion,** mode of action of isoprenaline on, in the conscious rat, 17
**Gastrin,** effect of metiamide on acid secretion stimulated by, in isolated whole rat stomach, 149
**Gastrocnemius muscle,** effect of corticosteroids on responses of, to nerve stimulation in the pithed rat, 302P
**Glucocorticoids,** effect of, on turnover of acetylcholine and prostaglandins in isolated guinea-pig ileum, 475P
**Glucose,** effect of some receptor antagonists on fenfluramine-induced uptake of, into isolated rat hemidiaphragm, 286P
**Glucose utilization,** inhibition of, in adipose tissue of man and rat, by amphetamine-type anorectic drugs, 436P
**Glutamate,** responses evoked by, from lobster muscle fibre and frog spinal cord, effect of antihistamines on, 583
**L-Glutamate,** effect of, on rat and frog spinal neurones, 373
—, anaesthetics depress sensitivity of cortical neurones to, 347
**Glutamate antagonists,** in rat hippocampus, 341
**Glutamic acid,** compounds related to, structure-activity relations of, on frog and rat spinal neurones, 373
**Glutamic acid dimethyl ester,** effect of, on rat hippocampal cells, 341
**Glycosides,** cardiac, role of potassium in inhibition by, of (Na–K)-ATPase prepared from human heart, 413P
**Guanethidine,** effect of TEA on response of rat anococcygeus muscle to, 47
—, extra-vesicular binding of, in adrenergic neurones, 497
—, effect of, on cardiovascular effects of dopamine in conscious cat, 505
**Guanosine triphosphate,** increases incidence of ethanol withdrawal head twitches in mice, 9

H

**$H_1$ antagonists,** effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583
**$H_2$ antagonists,** effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583
**$H_1$- and $H_2$-receptors,** effects of activation of, on central cardiovascular structures in cats and on behaviour in chickens, 442P
**Haloperidol,** effect of, on cardiovascular effects of dopamine and α-methyldopamine in conscious cat, 505
—, comparative studies on effects of, with those of metoclopramide on the EEG in conscious rat, 269P

—, comparison of effects of, with those of buprenorphine on concentrations of HVA, 5-HIAA and MHPG in rat forebrain, 275P
—, effect of, on fenfluramine-induced glucose uptake into isolated rat hemidiaphragm, 286P
**Halothane,** change in sensitivity to, in the rat following administration of sodium barbitone in the drinking water, 285P
—, determination of sleeping time induced by, in the rat, 27
—, effect of, on sensitivity of cortical neurones to acetylcholine, 359
—, effect of, on sensitivity of cortical neurones to L-glutamate, 347
**Headache,** drug-induced, behavioural changes in rats suggesting, 439P
**Heart,** atrial and ventricular contractile tissue of, heterogeneous reactivity of, 409P
—, autonomous control of, in canine ischaemic myocardium, 410P
—, human, (Na–K)-ATPase prepared from, role of potassium on inhibition by cardiac glycosides of, 413P
**Heart rate,** effect of dopamine on, in conscious cat, 505
—, effect of α-methylnoradrenaline, adrenaline and noradrenaline on, after administration into area of rat nucleus tractus solitarii, 593
**Heidenhain pouch,** effect of isoprenaline on aspects of gastric secretion in the rat with, 17
**Hexachloroethane,** action of, and products of metabolism in sheep on *Fasciola hepatica*, 395
**Hexamethonium,** effect of, on inhibitory innervation of cat sphincter of Oddi, 477
**Hexamethonium bromide,** effect of, on cardiovascular effects of dopamine in conscious cat, 505
**Hippocampus,** rat, glutamate antagonists in, 341
**Histamine,** release of, dopaminergic modulation of, in calf, 445P
—, release of, inhibition of, from rat peritoneal mast cells by non-steroid anti-inflammatory agents, 446P
—, effect of, on responses evoked by amino acids from lobster muscle fibre and frog spinal cord, 583
—, coronary vascular response to, 89
—, dose-response curves in guinea-pig denervated cremaster muscle preparation, 229
—, effect of indomethacin and prostaglandin on smooth muscle contracting activity of, 223
—, effect of isoprenaline on secretion of gastric mucosal, 17
—, effects of, on cat lung strip preparation, 71
**Histamine H$_1$-blockers,** effect of, on frog spinal cord and lobster muscle fibre, 583
**Histamine receptors,** in cranial circulation of monkey, 443P
**DL-Homocysteate,** effect of, on rat and frog spinal neurones, 373
**Homovanillic acid,** effect of acute doses of buprenorphine on concentrations of, in rat forebrain, 275P
**Human serum albumin,** effect of endogenous metabolites on binding of *o*-methyl red to, 283P
**6-Hydroxy-2-pyridylalanine,** effect of, on rat and frog spinal neurones, 373
**5-Hydroxyindoleacetic acid,** metabolism of, in brain, effect of aminophylline on, 533
—, effect of acute doses of buprenorphine on concentrations of, in rat forebrain, 275P
**5-Hydroxytryptamine,** high affinity binding sites to brain synaptosomal membranes, 425P
—, inhibition of stimulant effect of, on cardiac sympathetic nerves by 5-HT and related compounds, 416P
—, effect of, on effect of *N*-2-chloroethyl-*N*-ethyl-2-bromobenzylamine hydrochloride on rat brain and heart, 521
—, metabolism of, in brain, effect of aminophylline on, 533
—, comparison of D and L-tryptophan on cerebral metabolism of, and dopamine in the dog, 3
—, effect of, as substrate on monoamine oxidase activity in rat brain and spinal cord, 279P
—, effect of anorectic drugs on uptake and release of, in the brain, 272P
—, effects of aspirin and indomethacin on release of, by rat platelets induced by collagen and arachidonic acid, 299P
—, effect of, on arachidonic acid-induced paw oedema in the rat, 311P
—, effect of mazindol on metabolism of, in rat brain, 271P
—, effects of, on rabbit isolated ear artery and aorta, 211
—, increases release of transmitter from rabbit cardiac sympathetic nerves, 276P
—, mechanism of entry into rabbit erythrocytes, 294P
—, receptors for, in rabbit isolated ear artery and aorta, 211
—, transport of, by rat and human platelets, 300P
**[$^{14}$C]-5-Hydroxytryptamine,** uptake of, by platelets, in emotional and non-emotional rats, 454P
**[$^{3}$H]-5-Hydroxytryptamine,** selective accumulation of, by supra-ependymal nerve terminals, autoradiographic evidence for, 424P
**Hyoscine,** decreases incidence of ethanol withdrawal head twitches in mice, 9
**Hyperlactataemia,** acute experimental, pharmacological agents and, in dog, 429P
**Hypertension,** mineralocorticoid, evolution of cyclic AMP phosphodiesterase during onset of, in rat, 434P
—, renal prostaglandins and renal function during experimental, in rats, 253
—, Okamoto spontaneous, altered reactivity of stomach fundus smooth muscle in, 432P
**Hypertensive rats,** genetic, prostaglandin E$_2$ released from kidneys of, contributes to vasoconstrictor supersensitivity to angiotensin II *in vitro*, 462P
**Hypotension,** in rabbits infected with *Trypanosoma brucei*, 137
—, centrally induced, after administration of α-methylnoradrenaline into area of nucleus tractus solitarii of rat, 593
**Hypothermia,** comparison between, induced by thyrotropin releasing hormone, noradrenaline, or calcium ions in cats, 489
**Hypoxia,** resistance to, effects of carbonic anhydrase inhibitors on, 417P

I

**Ibuterol,** effects of, a new bronchodilator, 43
**Ileum,** kinetics of competitive antagonists on guinea-pig, 57
—, guinea-pig, comparison of affinity constants for muscarine-sensitive acetylcholine receptors in, 613
**Immunological challenge,** of guinea-pig lungs, prostaglandin-like substances released from, 471P
**Indapamide,** studies on the marked antihypertensive properties of, in rats and cats, 282P
**Indole derivatives,** administration of, a comparison of circling behaviour induced in nigro-striatal lesioned rats after, 452P

**Indolizine,** an, with an amiodarone-like haemodynamic profile, 411P
**Indomethacin,** and acid secretion in isolated perfused canine stomach, 470P
—, effectiveness of, against inflammation, 573
—, effect of, on effect of N-5′, 483
—, effect of, on arachidonic acid-induced paw oedema in the rat, 311P
—, effects of, on extent and duration of malondialdehyde production by rat platelets, 299P
—, effects of, on 5-HT release induced by arachidonic acid and collagen in rat platelets, 299P
—, effect of, on smooth muscle contracting activity of angiotensin and other agonists, 223
—, inhibits release of prostaglandin stimulated by bradykinin or kininogen in rabbit perfused kidney, 165
**Indoxyl sulphate,** effect of, on binding of *o*-methyl red to human serum albumin, 283P
**Inflammation,** the polymorph transfer reaction as a model system for the study of acute, 310P
**Inflammatory exudates,** non-immunological, mitogenic activity of different types of, on macrophages in culture, 448P
—, new method of obtaining human skin, for pharmacological analysis, 317P
**Innervation,** inhibitory, of cat sphincter of Oddi, 477
**Intracerebral administration,** a simple and inexpensive method for, of drug solutions to conscious rat, 464P
**Islets of Langerhans,** isolated, non-saturable co-operative binding of tolbutamide to, 450P
**3-Isobutyl-1-methyl-xanthine,** increases incidence of ethanol withdrawal head twitches in mice, 9
**Isoprenaline,** chronic pretreatment with, affects sensitivity of guinea-pig lung adenylate cyclase to isoprenaline and noradrenaline *in vitro,* 274P
—, contractual response of denervated mouse diaphragm muscle to, 109
—, coronary vascular response to, 89
—, effects of, on cat lung strip preparation, 71
—, effect of, on growth and secretion in rat parotid gland, 281P
—, effect of, on guinea-pig urinary bladder, 37
—, mode of action of, on gastric secretion in the conscious rat, 17
—, response of denervated cremaster muscle preparation to, 229
—, long-term culture with, dose-dependent desensitization of lymphocyte beta-receptor function after, 448P
—, evidence of central cardiovascular effects of intracerebroventricular, in anaesthetized rat, 435P
—, effect of, on cardiovascular effects of dopamine in conscious cat, 505
**Isoxsuprine,** effects of, on guinea-pig atria and trachea, 431P

K

**Kainate,** effect of, on rat and frog spinal neurones, 373
**Kallikrein,** not the sole mediator of parasympathetic vasodilatation in the dog salivary gland, 201
**Ketamine,** effects of, on the peripheral autonomic nervous system of the rat, 247
**Kinin,** not the sole mediator of parasympathetic vasodilatation in the dog salivary gland, 201
**Kininogen,** effect of, on renal prostaglandin release in rabbit perfused kidney, 165
**Kinins,** possible influence of intrarenal generation of, on PG release from rabbit perfused kidney, 165

L

**Lachesine,** kinetics of, in guinea-pig ileum, 57
**Langendorff perfusion,** of isolated rabbit heart, 316P
**Lauric acid,** effect of, on binding of *o*-methyl red to human serum albumin, 283P
**Lesions,** nigro-striatal, in rats, circling behaviour induced by, after administration of indole derivatives, 452P
**Lignocaine,** effect of, on cardiovascular effects of dopamine in conscious cat, 505
**Lipolysis,** catecholamine-induced, inhibition of, by amphetamine-type anorectic drugs, 436P
**$\beta$-Lipotropin,** interaction of peptides derived from C-fragment
**Liquor space,** analgesia produced by morphine when acting from, 383
**LSD,** high affinity binding sites to brain synaptosomal membranes, 425P
**$[^3H]$-D-LSD,** localization of binding sites for, autoradiographic evidence for, 424P
**Lymphocyte beta-receptor function,** dose-dependent desensitization of, after long-term culture with isoprenaline, 448P
**Lymphocytes,** role of, in accumulation of fibrin in rabbit skin homografts, 292P
**Lynestrenol,** effect of, combined with mestranol on drug metabolism, 173

M

**MA 1420,** decreases incidence of ethanol withdrawal head twitches in mice, 9
**$\alpha_1$-Macroglobulin,** effect of cortisol on, in arthritic rat, 441P
**Macrophages,** in culture, mitogenic activity of non-immunological inflammatory exudates on, 448P
**Malaria,** studies on *Azadiracta indica* in, 318P
**Malondialdehyde,** effects of aspirin and indomethacin on production of, by rat platelets, 299P
**Mazindol,** effect of, on uptake and release of brain monoamines, 272P
—, some effects of, on metabolism of monoamines in the rat brain, 271P
**Mazindol anorexia,** is mediated by activation of dopaminergic mechanisms, 367
**Medicines evaluation and monitoring,** Aberdeen–Dundee group, 317P
**Meprobamate,** effect of pretreatment with, on halothane-induced sleeping time in rats, 27
**Mepyramine,** affinity constant of guinea-pig cremaster muscle for, 229
—, effect of, on arachidonic acid-induced paw oedema in the rat, 311P
—, effect of, on fenfluramine-induced glucose uptake into isolated rat hemidiaphragm, 286P
—, kinetics of, in guinea-pig ileum, 57
**Mestranol,** effect of, in combination with lynestrenol, norethisterone or norethynodrel on drug metabolism in various animal species, 173

**Met-enkephalin,** effects of, on nociceptive neurones in rat thalamus, 459P
**Metaffinoid antagonism,** as term used for effect of gallamine on muscarinic receptors, 323
**Methamphetamine,** effect of, on suppressed responding, 451P
**Methergoline,** effect of, on mazindol anorexia, 367
—, decreases incidence of ethanol withdrawal head twitches in mice, 9
**L-Methionine,** effects of, on activity in the rat, 117
**3-Methoxy-4-hydroxyphenylglycol,** effect of acute doses of buprenorphine on concentrations of, in rat forebrain, 275P
**Methoxamine,** effect of denervation and cocaine on response of isolated rat vas deferens to, 280P
**Methoxyflurane,** effect of, on sensitivity of cortical neurones to acetylcholine, 359
—, effect of, on sensitivity of cortical neurones to L-glutamate, 347
**Methoxyverapamil,** see D600, 287P
***N*-Methyl-D-aspartate,** effect of, on rat and frog spinal neurones, 373
***N*-Methyl-5-HT,** stimulates release of transmitter from rabbit cardiac sympathetic nerves, 276P
**Methyl red,** effect of endogenous metabolites on binding of, to human serum albumin, 283P
**α-Methyl-*p*-tyrosine,** effect of, on mazindol anorexia, 367
—, effect of, on effect of amantadine on brain concentrations of dopamine and noradrenaline, 561
**α-Methyldopamine,** cardiovascular effect of, in conscious cat, 505
**α-Methylnoradrenaline,** centrally induced hypotension and bradycardia after administration of, into area of nucleus tractus solitarii of rat, 593
**α-Methyltryptophan,** effect of, on rat social behaviour, 455P
**α-Methyltyrosine,** effect of, on hypothermia induced in unanaesthetized cat, 489
**Methysergide,** decreases incidence of ethanol withdrawal head twitches in mice, 9
—, effect of, in combination with triprolidine on arachidonic acid-induced paw oedema in the rat, 311P
—, effect of, on fenfluramine-induced glucose uptake into isolated rat hemidiaphragm, 286P
—, mode of action of, in treatment of migraine and effect on isolated rabbit ear artery and aorta, 211
**Methylxanthines,** effect of, on aromatic amino acids in plasma, brain and other tissues, and on brain 5-HT metabolism, 533
**Metiamide,** action of, in anaphylaxis *in vivo* in the guinea-pig, 303P
—, effect of, on acid secretion stimulated by gastrin, acetylcholine and dibutyryl cyclic AMP in isolated whole rat stomach, 149
—, effect of, on response of denervated cremaster muscle to histamine, 229
**Metoclopramide,** comparative studies on effects of, and some known neuroleptics on the EEG of the conscious rat, 269P
**Migraine,** interactions of drugs used in treatment of, with 5-HT and noradrenaline in rabbit isolated ear artery and aorta, 211
**Milk-ejection reflex,** central and peripheral inhibition of, studies with β-adrenoceptor antagonists, 464P
**Mimosine,** effect of, on rat and frog spinal neurones, 373
**Mineralocorticoid hypertension,** evolution of aortic and cardiac cyclic AMP phosphodiesterase during the onset of, in rat, 434P
**Mitogenic activity,** of different types of non-immunological inflammatory exudates on macrophages in culture, 448P
**Monoamine oxidase activity,** and carbohydrate metabolism, 450P
—, effect of subcutaneous adrenaline injections on platelet activity, 291P
—, studies on, in rat spinal cord, 279P
**Monoamines,** some effects of mazindol on metabolism of, in the rat brain, 271P
**Morphine,** actions of, on spinal cord and brain stem neurones, 458P
—, effects of, on nociceptive neurones in rat thalamus, 459P
—, effect of, on priming-induced audiogenic seizures in BALB/c mice, 517
—, analgesia produced by, when acting from the liquor space, 383
—, effect of, on inhibitory innervation of cat sphincter of Oddi, 477
—, comparison of self-administration liability of, with pentazocine in the rhesus monkey, 270P
—, interaction between, and naloxone in central cholinergic system, 101
**Muscarine,** acetylcholine receptors in guinea-pig atrial pacemaker cells and ileum sensitive to, comparison of affinity constants for, 613
**Muscarinic receptors,** inhibitory effect of gallamine on, 323
—, in rat brain, ontogenesis of, 427P
**Muscle,** mammalian skeletal, effect of adrenaline on isotonic contractions of, 467P
—, smooth, stomach fundus, altered reactivity of, in Okamoto spontaneous hypertension, 432P
**Muscle fibre,** lobster, antagonism by some antihistamines of amino acid-evoked responses from, 583
**Myocardial infarction,** acute, in dog, effects of dobutamine on early stages of, 407P
—, experimental study of arrythmias following, in closed-chest dog, its use as a test for antiarrythmic drugs, 409P
**Myocardial strips,** action of cytochrome C on trans-membrane potentials of normal and hypoxic guinea-pig, 431P
**Myocardium,** uptake of digoxin in chronically digitalized dog, 553
—, canine ischaemic, atenolol, regional myocardial blood flow and S-T segement in, 411P
—, canine ischaemic, autonomous control of heart, myocardial blood flow and S-T segment in, 410P
**Myristic acid,** effect of, on binding of *o*-methyl red to human serum albumin, 283P

N

**N-0164** *see* Sodium *p*-benzyl-4-(1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl)phenyl phosphonate, 33
**Naloxone,** effect of, on priming-induced audiogenic seizures in BALB/c mice, 517
—, evidence for, and opiates as GABA antagonists, 458P
—, effect of, on response of rat forebrain to buprenorphine or haloperidol, 275P
—, interaction between, and morphine in central cholinergic system, 101

**Nandrolone,** effect of, on putrescine metabolism in mouse, 401

**Nephrectomy,** molecular mechanism of uterine sensitivity to angiotensin after, 433P

**Nerve fibres,** tryptaminergic, theory of morphine sensitivity of, 383

**Nerve terminals,** supra-ependymal, effects of specific uptake inhibitors on selective accumulation of [$^3$H]-5-HT by, autoradiographic evidence for, 424P

**Nerves,** cardiac sympathetic, inhibition of stimulant effect of 5-HT on, by 5-HT and related compounds, 416P

**Neuromuscular block,** disposition of labelled decamethonium in cat muscle, 141

**Neurones,** rat and frog spinal, effect of excitatory amino acids on, 373

—, spinal cord and brain stem, actions of morphine and enkephalin on, 458P

—, nociceptive, in rat thalamus, effects of met-enkephalin and morphine on, 459P

—, cortical, anaesthetics depress sensitivity of, to L-glutamate, 347

—, noradrenergic, in rat brain and heart, effect of $N$-2-chloroethyl-$N$-ethyl-2-bromobenzylamine hydrochloride on, 521

—, cortical, general anaesthetics and the acetylcholine sensitivity of, 359

**Neurotransmitter release,** and uptake, functional coupling of, in brain, 569

**Neurotransmitters,** putative amine, effect of $p$-chloro-$\alpha$-phenylethylamine on uptake and release of, in rat brain, 420P

**Nialamide,** effect of, on binding and efflux characteristics of noradrenaline and guanethidine, 497

**Nicotine,** decreases incidence of ethanol withdrawal head twitches in mice, 9

**Nifedipine,** effects of, on electrical and mechanical activity in rat portal vein, 287P

**Nitroglycerin,** headache induced by, behavioural changes in rats suggesting, 439P

**Noradrenaline,** metabolism of, in discrete areas of brain, modification by oestrogen of effects of D-amphetamine sulphate on, 422P

—, chronic pretreatment with bronchodilators affects sensitivity of guinea-pig lung adenylate cyclase to, 274P

—, comparison of effects of, with those of DOPA on single cortical neurones, 239

—, contractions evoked by, not inhibited by clonidine, 278P

—, decreases incidence of ethanol withdrawal head twitches in mice, 9

—, effect of anorectic drugs on uptake and release of, in the brain, 272P

—, effect of denervation and cocaine on response of isolated rat vas deferens to, 280P

—, effect of ketamine on motor responses to, in anococcygeus muscle, 247

—, effects of, on cat lung strip preparation, 71

—, effect of mazindol on metabolism of, in rat brain, 271B

—, effect of PGE$_1$ on inhibition induced by, in guinea-pig ileum, 290P

—, effect of, on guinea-pig urinary bladder, 37

—, effects of, on rabbit isolated ear artery and aorta, 211

—, influence of, on isolated cerebral arteries of the dog, 121

—, lack of effect of oxytetracycline on constriction of rabbit ear arteries induced by, 261

—, mechanism of entry into rabbit erythrocytes, 294P

—, pressor response to, reduced by indapamide in rats and cats, 282P

—, receptors for, in rabbit isolated ear artery and aorta, 211

—, released at nerve endings together with dopamine-$\beta$-hydroxylase, 275P

—, response of denervated cremaster muscle preparation to, 229

—, centrally induced hypotension and bradycardia after administration of, into area of rat nucleus tractus solitarii, 593

—, further evidence that prostaglandins inhibit release of, from adrenergic nerve terminals by restriction of availability of calcium, 599

—, intracarotid injection, long-lasting effects of opening of blood-brain barrier on modifications induced by, 417P

—, hypothermia induced by, in unanaesthetized cat, 489

—, extra-vesicular binding of, in adrenergic neurones, 497

—, effect of amantadine on brain concentration of, 561

**[$^3$H]-(−)-Noradrenaline,** effects of AH 5158 on metabolism of, released from cat spleen by nerve stimulation, 280P

**Norethisterone,** effect of, combined with mestranol on drug metabolism, 173

**Norethynodrel,** effect of, combined with mestranol on drug metabolism, 173

**Norfenfluramine,** effect of, on uptake and release of brain monoamines, 272P

**Nucleus accumbens,** heterogeneity of inhibitory mechanisms in the rat, 267P

**Nucleus tractus solitarii,** rat, centrally induced hypotension and bradycardia after administration of $\alpha$-methylnoradrenaline into area of, 593

O

**Obesity,** two models of, activity of anorectic drugs on, 437P

**Oedema,** induced in rat paw by arachidonic acid, 311P

**Oestrogen,** modification by, of effects of (+)-amphetamine sulphate on noradrenaline metabolisms in discrete areas of rat brain, 422P

**Opiates,** evidence for, as GABA antagonists, 458P

**Opiate receptors,** brain, interaction of peptides derived from C-fragment of $\beta$-lipotropin with, 460P

**Ouabain,** effect of, on contractual response of denervated mouse diaphragm to acetylcholine or isoprenaline, 109

—, effect of, on K$^+$-induced relaxations of the rat anococcygeus muscle, 305P

**Oxprenolol,** in angina pectoris, 412P

**Oxygen,** decrease in myocardial consumption of, following administration of chloracyzine, 189

**Oxygen availability,** cerebral cortex, effects of carbonic anhydrase inhibitors on, 417P

**Oxymetazoline,** without effect on growth and secretion in rat parotid gland, 281P

**Oxytetracycline,** lack of effect of, on responses to sympathetic nerve stimulation and catecholamines in vascular tissue, 261

P

**Pancreas,** rat exocrine, *in vitro,* effect of dibutyryl cyclic adenosine-3′,5′-monophosphate on protein secretion from, 605

**Paraquat,** *in vivo* absorption studies with, in the dog, 284P

**Parkinson's disease,** L-DOPA and (–)-deprenil in treatment of, 423P
**Parotid gland,** receptors involved in catecholamine-mediated growth and secretion in rat, 281P
**Pavlov pouch,** effect of isoprenaline on aspects of gastric secretion in the rat with, 17
**Pempidine,** effect of, on cardiovascular effects of dopamine in conscious cat, 505
**Pentachloroethane,** action of, and products of metabolism in sheep on *Fasciola hepatica*, 395
**Pentazocine,** comparison of self-administration liability of, with morphine in the rhesus monkey, 270P
**Pentobarbital sodium,** effect of, on suppressed responding, 451P
**Pentobarbitone,** effect of, on sensitivity of cortical neurones to acetylcholine, 359
—, effect of, on sensitivity of cortical neurones to L-glutamate, 347
—, effect of pretreatment with, on halothane-induced sleeping time in rats, 27
**Pentyl TEA,** kinetics of, in guinea-pig ileum, 57
**Pepsin,** effect of isoprenaline on secretion of, 17
**Peptides,** interaction of, derived from C-fragment of $\beta$-lipotropin with brain opiate receptors, 460P
**Perfusion pressure,** a device for controlling, 463P
**Perfusion techniques,** Langendorff mammalian heart perfusion, 316P
**Peripheral airways,** cat lung strip as an *in vitro* preparation of, 71
**Pethidine,** pharmacokinetics of, in dog, dose and time studies, 472P
**Pharmacology,** career patterns in science graduate pharmacologists, 309P
—, comparison of performance in examinations using multiple choice and essay questions, 306P
—, developments in the use of television for teaching, 314P
—, effective demonstrations to 100 students, 314P
—, evaluation of slide-tape programmes in the teaching of pharmacology, 315P
—, films in teaching of, 313P
—, learning from and attitudes to routine tape-slide teaching, 307P
—, teaching methods in, 316P
—, teaching to undergraduate pharmacy students, 308P
**Phenindamine,** effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583
**Phenmetrazine,** effect of, on uptake and release of brain monoamines, 272P
**Phenol,** effect of, on binding of *o*-methyl red to human serum albumin, 283P
**Phenoxybenzamine,** effect of, on release of noradrenaline from adrenergic nerve terminals, 599
—, effect of, on mazindol anorexia, 367
—, effect of, on inhibitory innervation of cat sphincter of Oddi, 477
—, effect of, on guinea-pig urinary bladder, 37
**Phentermine,** effect of, on uptake and release of brain monoamines, 272P
**Phentolamine,** effect of, on cardiovascular effects of $\alpha$-methylnoradrenaline after administration into area of rat nucleus tractus solitarii, 593
—, effect of, on hypothermia induced in unanaesthetized cat, 489
—, effect of, on cardiovascular effects of dopamine and $\alpha$-methyldopamine in conscious cat, 505
—, anatagonizes responses of single cortical neurones to noradrenaline and DOPA, 239
—, effect of, on contractual response of denervated mouse diaphragm to acetylcholine or isoprenaline, 109
—, effect of, on isolated rabbit ear artery and aorta, 211
—, effect of TEA on response of rat anococcygeus muscle to, 47
**Phenylalanine,** in plasma, brain and other tissues, effect of aminophylline on, 533
**Phenylbutazone,** effect of, on effect of N-5′, 483
—, effect of, on arachidonic acid-induced paw oedema in the rat, 311P
**Phenylethylamine,** formation of, in perfused rat liver, 474P
**Phospholipase A$_2$,** effect of an inhibitor of, on prostaglandin production by guinea-pig uterus, 295P
**Photostimulation,** iterative, decremental skin conductance response in mice during, an attention sustaining capacity model for psychopharmacological research, 437P
**Physostigmine,** increases incidence of ethanol withdrawal head twitches in mice, 9
**Pimozide,** effect of, on cardiovascular effects of dopamine and $\alpha$-methyldopamine in conscious cat, 505
—, effect of, on mazindol anorexia, 367
—, comparative studies on effects of, with those of metoclopramide on the EEG in conscious rat, 269P
**Pizotifen,** effect of, on isolated rabbit ear artery and aorta, 211
**Platelets,** blood, active transport of adenine and adenosine by, 466P
—, rabbit, plasma-free, effects of prostaglandin synthetase inhibitors on aggregation of, by carrageenin and thrombin, 447P
—, effects of aspirin and indomethacin on malondialdehyde production and the release reaction in rat, 299P
—, effect of pulmonary prostaglandin metabolites on ADP-induced aggregation of, in humans and rabbit, 297P
—, uptake by, of [$^{14}$C]-5-HT in emotional and non-emotional rats, 454P
—, effect of subcutaneous injections on adrenaline, on MAO activity in, 291P
—, stimulation of, by bis-enoic prostaglandins, 298P
—, transport of 5-HT by rat and human, 300P
**Polymorphonuclear leukocytes,** generation of thromboxane A$_2$-like activity from prostaglandin endoperoxides by, 296P
**Polymorph transfer reaction,** a model system for the study of acute inflammation, 310P
**Positive inotropic effect,** activity dependent prolongation of, by batrachotoxin, 415P
**Post-nephrectomy,** uterine supersensitivity, to angiotensin, molecular mechanism of, 433P
**Potassium,** role of, in inhibition by cardiac glycosides of (Na-K)-ATPase prepared from human heart, 413P
—, effect of, on release of GABA from isolated synaptosomal fraction from mouse forebrain, 569
—, myocardial, effect of digoxin, in dog, 553
—, effects of high rates of vasopressin administration on renal excretion of, 81
—, myocardial loss of, following administration of digoxin, 183
—, relaxations of the rat anococcygeus muscle induced by, 305P
**Potassium chloride,** effect of, on guinea-pig cremaster muscle preparation, 288P

**Potentials,** transmembrane, of normal and hypoxic guinea-pig myocardial strips, action of cytochrome C on, 431P

**Practolol,** effects of, on cat lung strip preparation, 71

**Prednisolone,** effect of, on effect of N-5′, 483

—, effect of, on neuromuscular function in the pithed rat, 302P

**Preoptic area,** heterogeneity of inhibitory mechanisms in the rat, 267P

**Procaine,** effect of, on cardiovascular effects of dopamine in conscious cat, 505

—, effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583

—, effect of, on K$^+$-induced relaxations of the rat anococcygeus muscle, 305P

**Proline,** effect of, on rat hippocampal cells, 341

**Propranolol,** long-term effects of, on tyrosine hydroxylase and dopamine-$\beta$-hydroxylase in superior cervical ganglia of rabbit, 430P

—, effect of, on cardiovascular effects of dopamine in conscious cat, 505

—, effect of, on effect of aminophylline on aromatic amino acids in plasma, brain and other tissues, 533

—, effect of, on effect of gallamine on muscarinic receptors, 323

—, antagonizes responses of single cortical neurones to noradrenaline and dopa, 239

—, effects of, on cat lung strip preparation, 71

—, effect of, on contractual response of denervated mouse diaphragm to acetylcholine or isoprenaline, 109

—, effect of, on fenfluramine-induced glucose uptake into isolated rat hemidiaphragm, 286P

—, effect of, on guinea-pig urinary bladder, 37

—, effect of, on response of dog cerebral arteries to dopamine and noradrenaline, 121

**[$^3$H]-Propranolol binding,** characteristics and altered sensitivity of cerebral $\beta$-adrenoceptors assessed by, 435P

**(±)-Propranolol,** effect of, on recovery of urinary concentration process after frusemide in rat, 413P

**(−)-Propranolol,** effect of, on mazindol anorexia, 367

**Prophylactic action,** of topically applied sunscreen agents, 573

**Propyl gallate,** local antinociceptive and topical anti-inflammatory effect of, in rodents, 573

—, therapeutic value of, applied after u.v. irradiation, 573

**Prostaglandin,** synthesis of, catecholamine-stimulated, in rat brain synaptosomes, 426P

—, antagonism of, by sodium $p$-benzyl-4-(1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl)phenyl phosphonate (N-0164), 333

—, -like substances, biological significance of, released from immunologically challenged guinea-pig lungs, 471P

—, effect of $p$-bromophenacyl bromide on production of, by guinea-pig uterus, 295P

—, effect of, on smooth muscle contracting activity of angiotensin and other agonists, 223

**Prostaglandin endoperoxides,** generation of thromboxane $A_2$-like activity from, by polymorphonuclear leukocytes, 296P

**Prostaglandin synthetase inhibitors,** effects of, on aggregation of plasma-free rabbit platelets by carrageenin and thrombin, 447P

**Prostaglandins,** turnover of, effect of glucocorticoids on, in isolated guinea-pig ileum, 475P

—, further evidence that, inhibit release of noradrenaline from adrenergic nerve terminals by restriction of availability of calcium, 599

—, possible influence of intrarenal generation of kinins on release of, from rabbit perfused kidney, 165

—, renal function and renal venous concentrations of, during different stages of experimental renal hypertension in the rat, 253

—, stimulation of biosynthesis of, by various drugs, 193

—, stimulation of platelets by bis-enoic, 298P

**Prostaglandin E,** as a measure of prostaglandin synthesis induced by various drugs affecting gut function, 193

—, release of, from rabbit perfused kidney in response to kinins, 165

**Prostaglandin $E_1$,** decreases incidence of ethanol withdrawal head twitches in mice, 9

—, effect of chronic pretreatment with bronchodilators on sensitivity of guinea-pig lung adenylate cyclase to, 274P

—, effect of pulmonary metabolites of, on ADP-induced aggregation of human and rabbit platelets, 297P

—, postsynaptic action of, on sympathetic responses in guinea-pig ileum, 290P

**Prostaglandin $E_2$,** concentrations and secretion rates of, in renal hypertension in rats, 253

—, decreases incidence of ethanol withdrawal head twitches in mice, 9

—, effect of pulmonary metabolites of, on ADP-induced aggregation of human and rabbit platelets, 297P

—, inhibition of synthesis of, by guinea-pig uterus in presence of $p$-bromophenacyl bromide, 295P

—, released from kidneys of genetic hypertensive rats contributes to vasoconstrictor supersensitivity to angiotensin II *in vitro*, 462P

**Prostaglandins $E_2$ and $F_{2\alpha}$,** biosynthesis of, inhibited by propyl gallate in rodents, 573

**Prostaglandin $F_{2\alpha}$,** concentrations and secretion rates of, in renal hypertension in rats, 253

—, effects of, on cat lung strip preparation, 71

—, effect of pulmonary metabolites of, on ADP-induced aggregation of human and rabbit platelets, 297P

—, inhibition of synthesis of, in guinea-pig uterus in presence of $p$-bromophenacyl bromide, 295P

—, release of, from rabbit perfused kidney in response to kinins, 165

**Protein secretion,** effect of dibutyryl cyclic adenosine-3′,5′-monophosphate on, from rat exocrine pancreas *in vitro*, 605

**Putrescine,** metabolism in mouse, effect of nandrolone on, 401

Q

**Quisqualate,** responses evoked by, from lobster muscle fibre and frog spinal cord, effect of antihistamines on, 583

—, effect of, on rat and frog spinal neurones, 373

R

**Reserpine,** effect of, on accumulation of 5-HT by supra-ependymal nerve terminals, 424P

—, effect of, on binding and efflux characteristics of noradrenaline and guanethidine, 497

—, effect of, on mazindol anorexia, 367

—, effects of, in normal and adrenalectomized rats on neuromuscular function, 302P
**Respiration,** inhibition of, by amphetamine-type anorectic drugs, 436P
—, and blood pressure in the anaesthetized cat, 316P
**Response curves,** fitting a general sigmoid model to pharmacological, 289P
**Rubidium,** effect of, on release of noradrenaline from adrenergic nerve terminals, 599

S

**S-T segment,** autonomous control of, in canine ischaemic myocardium, 410P
—, atenolol, and regional myocardial blood flow in canine ischaemic myocardium, 411P
**Salbutamol,** effect of pretreatment with, on sensitivity of guinea-pig lung adenylate cyclase, 274P
—, effect of, on growth and secretion in rat parotid gland, 281P
**Salivary gland,** bradykinin and functional vasodilatation in, 201
**SE 1520,** see indapamide, 282P
**Secretin,** actions of natural, on small intestinal vasculature of anaesthetized cat, 127
**Seizures,** audiogenic, priming-induced, effect of morphine and naloxone on, in BALB/c mice, 517
**Septicaemia,** human, shocked lung syndrome of, treatment of, 547
**Serine,** effect of, on rat and spinal neurones, 373
**Shocked lung syndrome,** of human septicaemia, treatment of, 547
**Skeletal muscle,** simple *in vitro* preparation of mammalian tissue exhibiting properties of slow tonic, 288P
**Skin conductance,** decremental, response, in mice, during iterative photostimulation; an attention-sustaining capacity model for psychopharmacological research, 437P
**Sleep,** of man, effect of 1,5-benzodiazepines, clobazam and triflubazam on, 439P
**Sleeping time,** determination of halothane-induced, in the rat, 27
**Small intestine,** actions of natural secretin on vasculature of, in anaesthetized cat, 127
**Social interaction,** in rats, effects of ethanol and chlordiazepoxide on, 465P
**Sodium,** fluid reabsorption dependent on, in rat isolated caudal epididymis, effect of amiloride on, 529
—, effects of high rates of vasopressin administration on renal excretion of, 81
**Sodium barbitone,** change in sensitivity to halothane in the rat following administration of, in drinking water, 285P
**Sodium *p*-benzyl-4-(1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl)phenyl phosphonate,** antagonism of prostaglandins by, 333
**Sodium cromoglycate,** a further action of, in reversal of reflex bronchoconstriction, 301P
**Sodium nitroprusside,** effects of, on electrical and mechanical activity in rat portal vein, 287P
**Soybean trypsin inhibitor,** pretreatment with, affects course of arachidonic acid-induced paw oedema in the rat, 311P
**Spermidine,** metabolism in mouse, effect of nandrolone on, 401
**Spermine,** metabolism in mouse, effect of nandrolone on, 401
**Sphincter of Oddi,** cat, inhibitory innervation of, 477
**Spinal cord,** frog, antagonism by some antihistamines of amino acid-evoked responses from, 583
**Sponge implantation test,** in rat as a model for screening anti-inflammatory activity, 472P
**SRS-A,** release of, dopaminergic modulation of, in calf, 445P
**Stomach,** isolated perfused canine, acid secretion and indomethacin in, 470P
**Streptozotocin,** effect of, on effect of aminophylline on aromatic amino acids in plasma, brain and other tissues, 533
**Striato-nigral pathway,** is it responsible for 'feed-back' control of dopamine release?, 272P
**Succinylcholine,** response of guinea-pig cremaster nerve-muscle preparation to, 288P
**Sulphadimidine,** intersubject variability of acetylation of, in student volunteers, 312P
***O*-Sulpho-L-serine,** effect of, on rat and frog spinal neurones, 373
**Sunscreen agents,** topically applied, prophylactic action of, 573
**Superior cervical ganglion,** action of SGTX on nicotinic receptors in rat, 157
**Suppressed responding,** effects of drugs on, 451P
**Surugatoxin,** action of, on nicotinic receptors in superior cervical ganglion of the rat, 157
**Synaptosomes,** effect of amantadine on dopamine uptake by, 561
—, rat brain, catecholamine-stimulated prostaglandin synthesis in, 426P
**Synaptosomal fractions,** isolated, from mouse forebrain, neurotransmitter uptake and release in, 569
**Synaptosomal membranes,** brain, 5-HT and LSD high affinity binding sites to, 425P

T

**Tablet formulation,** class experiment illustrating influence of, on absorption of aspirin, 312P
**Tachycardia,** vasodilatory response to, 89
**Teaching methods,** class experiment with student volunteers illustrating influence of formulation on absorption of aspirin, 312P
—, comparison of performance in pharmacology examinations using multiple choice and essay questions, 306P
—, developments in the use of television for pharmacology teaching, 314P
—, effective pharmacological demonstrations to 100 students, 314P
—, films in pharmacology teaching, 313P
—, how can multiple choice examinations be set which are of equal difficulty from year to year, 305P
—, in pharmacology, 316P
—, intersubject variability of sulphadimidine acetylation in student volunteers, 312P
—, learning from and attitudes to routine tape-slide teaching, 307P
—, slide-tape programmes in the teaching of pharmacology, 315P
—, teaching pharmacology to undergraduate pharmacy students, 308P

**Temperature**, effect of, on affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells and ileum, 613

**Terbutaline**, effects of ibuterol, a pro-drug to, 43

**Tetrachloroethane**, action of, and products of metabolism in sheep on *Fasciola hepatica*, 395

**Tetraethylammonium**, effect of, on release of noradrenaline from adrenergic nerve terminals, 599

**Tetramethylammonium chloride**, effect of, on cardiovascular effects of doamine in conscious cat, 505

—, action of, on rat anococcygeus muscle, 47

**Tetrodotoxin**, effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583

—, action of, and product of metabolism in sheep on *Fasciola hepatica*, 395

—, effect of, on inhibitory innervation of cat sphincter of Oddi, 477

—, effect of, on $K^+$-induced relaxations of the rat anococcygeus muscle, 305P

—, effect of, on response of guinea-pig cremaster muscle preparation to acetylcholine and succinylcholine, 288P

**Theobromine**, effect of, on aromatic amino acids in plasma, brain and other tissues and on brain 5-HT metabolism, 533

**Theophylline**, increases incidence of ethanol withdrawal head twitches in mice, 9

**Thrombin**, aggregation of plasma-free rabbit platelets by, effects of prostaglandin synthetase inhibitors on, 447P

**Thromboxane $A_2$**, generation of activity resembling that of, from prostaglandin endoperoxides by polymorphonuclear leucocyte homogenates, 296P

**Thymoxamine**, effect of, on fenfluramine-induced glucose uptake into isolated rat hemidiaphragm, 286P

**Thyrotropin releasing hormone**, hypothermia induced by, in unanaesthetized cat, 489

**Thyroxine sodium**, effect of, on hypothermia induced in unanaesthetized cat, 489

**Tolbutamide**, non-saturable co-operative binding of, to isolated islets of Langerhans, 450P

**Trichloroethylene**, effect of, on sensitivity of cortical neurones to acetylcholine, 358

—, effect of, on sensitivity of cortical neurones to L-glutamate, 347

**Tricyclic antidepressants**, effect of, on 5-HT uptake by platelets, 300P

**Triflubazan**, effect of, on sleep of man, 439P

**2,4,5-Trihydroxyphenylalanine**, effect of, on rat and frog spinal neurones, 373

**Triprolidine**, effect of, on lobster muscle fibre and frog spinal cord, responses of, to amino acids, 583

—, effect of, in combination with methysergide on arachidonic acid-induced paw oedema in the rat, 311P

**Triproline**, effect of, on arachidonic acid-induced paw oedema in the rat, 311P

***Trypanosoma brucei***, hypotension in rabbits infected with, 137

**Tryptamine**, evidence for receptors for, on cardiac sympathetic nerves, 276P

—, effects of, on mechanical and electrical activities of sinoatrial frog auricular trabeculae, 410P

—, stimulates release of transmitter from rabbit cardiac sympathetic nerves, 276P

**Tryptophan**, in plasma, brain and other tissues, effect of aminophylline on, 533

**D-Tryptophan**, effect of, on cerebral metabolism of 5-HT and dopamine in the dog, 3

**L-Tryptophan**, effects of, on activity in the rat, 117

—, effect of, on cerebral metabolism of 5-HT and dopamine in the dog, 3

**(+)-Tubocurarine**, affinity constants for, in guinea-pig innervated and denervated cremaster muscle, 229

**Tyramine**, effect of, as substrate on monoamine oxidase activity in rat brain and spinal cord, 279P

—, effect of TEA on response of rat anococcygeus muscle to, 47

—, mechanism of entry into rabbit erythrocytes, 294P

—, pressor response to, reduced by indapamide in rats and cats, 282P

**Tyrosine**, in plasma, brain and other itssues, effect of aminophylline on, 533

**Tyrosine hydroxylase**, long-term effects of propranolol on, in superior cervical ganglia of rabbit, 430P

## U

**UP507-04**, activity of, on two models of obesity in animals, 437P

**Uptake$_1$ or $_2$ blockade**, effect of, on release of noradrenaline from adrenergic nerve terminals, 599

**Urea**, effect of, on binding of *o*-methyl red to human serum albumin, 283P

**Uric acid**, effect of, on binding of *o*-methyl red to human serum albumin, 283P

**Urinary bladder**, adrenoceptors of the guinea-pig, 37

**Urinary concentration process**, effect of (±)-propranolol on, after frusemide in rat, 413P

**Uterine supersensitivity**, post-nephrectomy, to angiotensin, molecular mechanism of, 433P

## V

**Vagotomy**, effect of, on cardiovascular effects of $\alpha$-methylnoradrenaline after administration into area of rat nucleus tractus solitarii, 593

**Vas deferens**, effects of denervation and cocaine on response of isolated rat, to noradrenaline and methoxamine, 280P

**Vasoconstrictor supersensitivity**, to angiotensin II *in vitro*, prostaglandin $E_2$ released from kidneys of genetic hypertensive rats contributes to, 462P

**Vasopressin**, effect of high rates of administration of, on renal potassium and sodium excretion during potassium loading in the sheep, 81

**Verapamil**, effect of, on hypothermia induced in unanaesthetized cat, 489

## X

**Xylocaine**, effect of, on hypothermia induced in unanaesthetized cat, 489

# British Journal of Pharmacology

EDITED FOR

THE BRITISH PHARMACOLOGICAL SOCIETY

BY

A. W. Cuthbert *(Chairman)* H. M. Adam *(Secretary)*
Margaret Day *(Press Editor)*
J. D. Stephenson *(Assistant Press Editor)*

A. M. Barrett
W. G. Bisset
T. B. Bolton
D. A. Brown
D. A. Callingham
T. J. Crow
F. de Matteis
C. T. Dollery
V. Eisen
E. W. Gill
A. F. Green
D. F. Hawkins
Margarethe Holzbauer
D. B. Hope
J. S. Kelly
G. M. Lees
G. P. Lewis
I. H. M. Main
E. K. Matthews
J. F. Mitchell
P. A. Nasmyth
M. W. Parkes
M. Sandler
A. P. Silverman
M. A. Simmonds
F. M. Sullivan
A. Ungar
R. A. Webster

VOLUME 58, 1976

LONDON
MACMILLAN JOURNALS LIMITED
4 LITTLE ESSEX STREET, WC2R 3LF

# British Journal of Pharmacology

## CONTENTS

### VOLUME 58 NUMBER 1 SEPTEMBER 1976

3 BUDNY, J., DOW, R.C., ECCLESTON, D., HILL, A.G. & RITCHIE, ISOBEL M. A comparison of D and L-tryptophan on the cerebral metabolism of 5-hydroxytryptamine and dopamine in the dog

9 COLLIER, H.O.J., HAMMOND, M.D. & SCHNEIDER, C. Effects of drugs affecting endogenous amines or cyclic nucleotides on ethanol withdrawal head twitches in mice

17 LUNDELL, L., SVENSSON, S.E. & NILSSON, G. Further studies on the mode of action of isoprenaline on gastric secretion in the conscious rat

27 TURNBULL, M.J. & WATKINS, J.W. Determination of halothane-induced sleeping time in the rat: effect of prior administration of centrally active drugs

37 DAVE, K.C. & DHATTIWALA, A.S. Adrenoceptors of the guinea-pig urinary bladder

43 HÖGLUND, ELIN, WESTLING, H. & WHITE, T. Circulatory effects of oral and subcutaneous administration in normal subjects of a new bronchodilator ibuterol, a pro-drug to terbutaline

47 GILLESPIE, J.S. & TILMISANY, A.K. The action of tetraethylammonium chloride on the response of the rat anococcygeus muscle to motor and inhibitory nerve stimulation and to some drugs

57 ROBERTS, FIONA & STEPHENSON, R.P. The kinetics of competitive antagonists on guinea-pig ileum

71 LULICH, K.M., MITCHELL, H.W. & SPARROW, M.P. The cat lung strip as an *in vitro* preparation of peripheral airways: a comparison of $\beta$-adrenoceptor agonists, autacoids and anaphylactic challenge on the lung strip and trachea

81 BEAL, A.M. The effect of high rates of vasopressin administration on renal potassium and sodium excretion during potassium loading in the sheep

89 BROADLEY, K.J. The releas
dilator metabolite from the
perfused heart stimulated
histamine and electrical pacin
anoxia

101 JHAMANDAS, K. &
Morphine–naloxone interact
cholinergic system: the infl
lesioning and electrical stimula

109 EVANS, R.H. & SMITH, J.
cholamines on the influx
development of tension in
diaphragm muscle

117 TAYLOR, M. Effects of
methionine on activity in the r

121 TODA, N. Influence of
adrenaline on isolated cerebr

127 RICHARDSON, P.D.I. Th
secretin on the small intestin
anaesthetized cat

137 BOREHAM, P.F.L. &
Hypotension in rabbits infe
*soma brucei*

141 CREESE, R. & MACLA
Labelled decamethonium in ca

149 BUNCE, K.T., PARSONS,
NITA A. The effect of metian
stimulated by gastrin, acetylc
cyclic adenosine 3′,5′-mon
isolated whole stomach of the

**SHORT COMMUNICATIO**

157 BROWN, D.A. & GA
HAYASHI, E. & YAMA
surugatoxin on nicotinic rece
cervical ganglion of the rat

### VOLUME 58 NUMBER 2 OCTOBER 1976

165 COLINA-CHOURIO, J., McGIFF, J.C., MILLER, M.P. & NASJLETTI, A. Possible influence of intrarenal generation of kinins on prostaglandin release from the rabbit perfused kidney

173 BRIATICO, G., GUISO, G., JORI, A. & RAVAZZANI, C. Effects of contraceptive agents on drug metabolism in various animal species

183 STEINESS, EVA & VALEN
digoxin uptake: dissociatio
induced inotropism and
potassium

189 KISIN, I.E. On the decrease
consumption induced by chl

., McDONALD-GIBSON,
EED, S.A. Stimulation of pro-
esis by drugs: effects *in vitro* of
ng gut function
& SMAJE, L.H. Bradykinin
dilatation in the salivary gland
A, HUMPHREY, P.P.A. &
ceptors for 5-hydroxytryptamine
e in rabbit isolated ear artery and

EANNINE, MENDES, GUN B.,
MARIA E., PAIVA, A.C.M. &
EREZINHA B. Effect of
nd prostaglandin on the smooth
ing activity of angiotensin and

UREEN, EVINÇ, AKGÜN &
NE. The denervated cremaster
uinea-pig as a pharmacological

239 BEVAN, P., BRADSHAW, C.M. & SZABADI, E. The action of micro-electrophoretically applied L-3,4-dihydroxyphenylalanine (DOPA) on single cortical neurones

247 CLANACHAN, A.S. & McGRATH, J.C. Effects of ketamine on the peripheral autonomic nervous system of the rat

253 SMITH, G.W. & SOMOVA, LILIANA I. Renal function and renal venous prostaglandin concentrations during different stages of experimental renal hypertension in the rat

261 KALSNER, S. The lack of effect of oxytetracycline on responses to sympathetic nerve stimulation and catecholamines in vascular tissue

267P PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY, Dundee, 15–16 July, 1976

## OCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY
University of Dundee, 15th–16th July, 1976

.R. & PHILLIPS, S.W.
inhibitory mechanisms in the
and preoptic area of the rat
., MILLER, A.A. &
Comparative studies on the
pramide and some known
EEG of the conscious rat
MER, D. Comparison of the
abilities of morphine and
esus monkey
& SUGRUE, M.F. Some
ol on the metabolism of
rat brain
W., GARCIA-MUNOZ, M.,
M., TULLOCH, I.F. &
troduced by Ashcroft, G.W.).
pathway responsible for 'feed-
amine release?
RINDAST, M.R. The effects
n uptake and release of brain

, MARSHALL, P.B. &
ffect of chronic pretreatment
c bronchodilator drugs on
guinea-pig lung adenylate

MAR, P.W. & WALTER,
doses of buprenorphine on
novanillic acid (HVA), 5-
cid (5-HIAA) and 3-
ylglycol (MHPG) in the

LEACH, G.D.H. The
dopamine-β-hydroxylase
rmo- and hypertensive rats

276P FOZARD, J.R. & MOBAROK ALI, A.T.M. Evidence for tryptamine receptors on cardiac sympathetic nerves
278P IDOWU, O.A. & ZAR, M.A. Inhibitory effect of clonidine on a peripheral adrenergic synapse
279P GEORGE, A.J. & JONES, G.T. Studies on monoamine oxidase in rat spinal cord
280P DEHPOUR, A.R., GHAFFARPOUR, F., HOSSEINZADEH, K. & KHOYI, M.A. (introduced by Michal, F.) Effect of denervation and cocaine on the response of isolated rat vas deferens to noradrenaline and methoxamine
280P SUMMERS, R.J. & TILLMAN, JANET. The effects of AH 5158 on metabolism of $^3$H(—)noradrenaline released from the cat spleen by nerve stimulation
281P CORBETT, A.D., MAIN, BARBARA E., MUIR, T.C. & TEMPLETON, D. The receptors involved in catecholamine mediated growth and secretion in rat parotid gland
282P FINCH, L. & HICKS, P.E. Studies on the marked antihypertensive properties of indapamide (SE 1520) in rats and cats
283P BOWMER, C.J. & LINDUP, W.E. Effect of endogenous metabolites on the binding of *o*-methyl red to human serum albumin
284P BENNETT, P.N., DAVIES, D.S. & HAWKESWORTH, GABRIELLE M. *In vivo* absorption studies with paraquat and diquat in the dog
285P TURNBULL, M.J. & WATKINS, J.W. Change in sensitivity to halothane in the rat following administration of sodium barbitone in the drinking water

286P KIRBY, MARILYN J. & TURNER, P. Effect of some receptor antagonists on fenfluramine-induced glucose uptake into the isolated rat hemidiaphragm
287P JETLEY, M. & WESTON, A.H. Some effects of D600, nifedipine and sodium nitroprusside on electrical and mechanical activity in rat portal vein
288P DALE, M. MAUREEN & MUID, R. A simple *in vitro* preparation of mammalian tissue exhibiting properties of slow tonic skeletal muscle
289P DAVEY, A., MARRIOTT, A. & STOCKHAM, M.A. Fitting a general sigmoid model to pharmacological response curves
290P SANGER, G.J. & WATT, A.J. A postsynaptic action of prostaglandin $E_1$ on sympathetic responses in guinea-pig ileum
291P BOURNE, R.C., FRITH, C.D., OWEN, F. & RILEY, G.J. (introduced by Crow, T.J.). The effect of subcutaneous injections of adrenaline on platelet MAO activity
292P BACH, CATHERINE S. & JASANI, M.K. Role of lymphocytes in accumulation of fibrin in rabbit skin homografts
294P BLAKELEY, A.G.H. & NICOL, C.J.M. Accumulation of biogenic amines by rabbit erythrocytes
295P MITCHELL, SHEILA M., POYSER, N.L. & WILSON, N.H. Effect of *p*-bromophenacyl bromide, an inhibitor of phospholipase $A_2$, on prostaglandin production by the guinea-pig uterus
296P BUNTING, S., HIGGS, G.A. MONCADA, S. & VANE, J.R. Generation of thromboxane $A_2$-like activity from prostaglandin endoperoxides by polymorphonuclear leukocyte homogenates
297P WESTWICK, J. (introduced by Lewis, G.P.). The effect of pulmonary metabolites of prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ on ADP-induced aggregation of human and rabbit platelets
298P GORDON, J.L. & MacINTYRE, D.E. Stimulation of platelets by bis-enoic prostaglandins
299P GORDON, J.L., MacINTYRE, D.E. & McMILLAN, R.M. Malondialdehyde production and the release reaction in rat blood platelets: inhibition by aspirin and indomethacin *ex vivo*
300P GORDON, J.L. & OVERMAN, H.J. Transport of 5-hydroxytryptamine by rat and human platelets
301P JACKSON, D.M. & RICHARDS, I.M. A further action of sodium cromoglycate
302P GIBSON, A., POLLOCK, D. & SPENCE, J.C. The effects of corticosteroids on the responses of the anococcygeus and gastrocnemius muscles to nerve stimulation in the pithed rat
303P GOADBY, P. & LITTLE, M. ANN. The action of metiamide in anaphylaxis *in vivo* in the guinea-pig
304P REGOLI, D., ST-LOUIS, J. & the late PARK, W.K. Factors regulating the time-course of the relaxation of rabbit aorta strips after contraction by angiotensin II
305P GIBSON, A. & JA relaxations of the rat ar
305P FOSTER, R.W. How ca examinations of equal dif
306P HOULT, J.R.S. Compa pharmacology examinatio and essay questions
307P FOY, J.M. Learning from tape-slide teaching
308P FOY, J.M. & SENIOR, JU pharmacology to unde students
309P MARSHALL, P.B. & STE patterns in science gradua
310P FIRRELL, J.C., PECK, M (introduced by Lewis, transfer reaction: a model acute inflammation
311P COTTNEY, J., LEWIS, Arachidonic acid-induced
312P LUSCOMBE, D.K. & experiment with student influence of formulation
312P LISCOMBE, D.K. Intersubject variabil acetylation in student v
313P HOULT, J.R.S. Films
314P D'MELLO, A. & pharmacological demo
314P LEACH, G.D.H. De television for pharmac
315P COX, B., FOZARD, J tape programmes in the
315P FOY, J.M. & HOPKI and the eye
316P FOY, J.M. & SEN methods in pharmacol
316P LEACH, G.D.H. Lan perfusion
316P FOY, J.M. & McC pressure and respirati
317P BLACK, A. KOB HENSBY, C.N. & method of obtaining exudate for pharmac
317P WEIR, R.D., MOI GALLON, S.C., DA & CHRISTOPHE medicines evaluatio
318P TELLA, A. Studi malaria

## VOLUME 58 NUMBER 3 NOVEMBER 1976

3 CLARK, A.L. & MITCHELSON, F. The inhibitory effect of gallamine on muscarinic receptors

33 EAKINS, K.E., RAJADHYAKSHA, V. & SCHROER, R. Prostaglandin antagonism by sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl]phenyl phosphonate (N-0164)

341 SEGAL, M. Glutamate antagonists in rat hippocampus

347 RICHARDS, C.D. & SMAJE, J.C. Anaesthetics depress the sensitivity of cortical neurones to L-glutamate

359 SMAJE, J.C. General anaesthetics and the acetylcholine-sensitivity of cortical neurones

367 KRUK, Z.L. & ZARRINDAST, M.R. Mazindol anorexia is mediated by activation of dopaminergic mechanisms

373 BISCOE, T.J., EVANS, R.H., HEADLEY, P.M., MARTIN, M.R. & WATKINS, J.C. Structure-activity relations of excitatory amino acids on frog and rat spinal neurones

383 DEY, P.K. & FELDBERG, W. Analgesia produced by morphine when acting from the liquor space

395 BARTLET, A.L. Actions of carbon tetrachloride, hexachloroethane and the products of their metabolism in sheep on *Fasciola hepatica*

401 HENNINGSSON, S. & ROSENGREN, ELSA. The effect of nandrolone, an anabolic steroid on putrescine metabolism in the mouse

407P PROCEEDINGS OF THE BRITISH AND FRENCH PHARMACOLOGICAL SOCIETIES, The University of Oxford, 15–17 September, 1976

## PROCEEDINGS OF THE BRITISH AND FRENCH PHARMACOLOGICAL SOCIETIES

The University of Oxford, 15th–17th September, 1976

407P MARSHALL, R.J. & PARRATT, J.R. The effects of dobutamine in the early stages of acute experimental myocardial infarction in the dog

408P DELAIGUE, R., DUCHENE-MARULLAZ, P., GUEORGUIEV, G. & KANTELIP, J.P. Comparative effects of chloralose and pentobarbital on a atrioventricular conduction in the dog

409P FARGES, J.P., FAUCON, G. & OLLAGNIER, M. Heterogeneous reactivity of atrial and ventricular cardiac contractile tissue

409P DUPUIS, B., GAUTIER, P., RICHER, G. & SERRE, M. A method for experimental study of arrhythmias following myocardial infarction in closed-chest dog. Its use as a test for antiarrhythmic drugs

410P GARNIER, D., GOUPIL, N., NARGEOT, J., POURRIAS, B. & ROUGIER, O. Effects of tryptamine on mechanical and electrical activities of sino-atrial frog auricular trabeculae

410P BERDEAUX, A., BOISSIER, J.R. & GIUDICELLI, J.F. Autonomous control of the heart, myocardial blood flow and S-T segment in canine ischaemic myocardium

411P BERDEAUX, A., BOISSIER, J.R. & GIUDICELLI, J.F. Atenolol, regional myocardial blood flow and S-T segment in canine ischaemic myocardium

411P CHARLIER, R.H., RAUTHIER, J.A. & RICHARD, J.C. An indolizine with an amiodarone-like haemodynamic profile

412P TAYLOR, S.H. & THADANI, U. Oxprenolol in angina pectoris

412P HARICHAUX, P. & HARY, L. Some complementary data on AH 5158, an inhibitor of both $\alpha$- and $\beta$-adrenoceptors

413P IMBS, J.L., SCHMIDT, M. & SCHWARTZ, J. Effect of (±)-propranolol on the recovery of urinary concentration process after frusemide, in the rat

413P DE POVER, A. & GODFRAIND, T. The role of potassium in the inhibition by cardiac glycosides of $(Na^+\text{-}K^+)$-ATPase prepared from human heart

415P HONERJÄGER, P. & REITER, M. (introduced by Trendelenburg, U.). Batrachotoxin: activity-dependent prolongation of the cardiac action potential and positive inotropic effect

416P FOZARD, J.R. & MOBAROK ALI, A.T.M. Inhibition of the stimulant effect of 5-hydroxytryptamine on cardiac sympathetic nerves by 5-hydroxytryptamine and related compounds

417P BOULU, R.G., LARSEN, R.G., PLOTKINE, M. & ROSSIGNOL, P. Effects of carbonic anhydrase inhibitors upon cerebral cortex oxygen availability and resistance to hypoxia

417P BOISMARE, F.R., HACPILLE, L., LEFRANCOIS, J.P. & LEPONCIN, M. Long-lasting effects of the opening of the blood brain barrier on the modifications induced by intracarotid injection of noradrenaline

418P BEVAN, P., BRADSHAW, C.M. & SZABADI, E. Neuronal responses to adrenoceptor agonists in the cerebral cortex: evidence for excitatory $\alpha$-adrenoceptors and inhibitory $\beta$-adrenoceptors

419P DESOIZE, B., JARDILLIER, J.C., LEGER, C. & NAHAS, G.G. Delta-9-tetrahydrocannabinol (THC) and macromolecular synthesis: mechanisms of action

419P PASCAUD, X.B. & ROGER, A.R. Is the gastric antisecretory property of clonidine in rats of central origin?

420P BAKER, G.B., BERTOLLINI, A., del CARMINE, R., MARTIN, I.L. & RAITERI, M. Effects of $p$-chloro-$\beta$-phenylethylamine on the uptake and release of putative amine neurotransmitters in rat brain

422P FLUDDER, JOAN M. & TONGE, SALLY R. (introduced by Leonard, B.E.). Modification by oestrogen of the effects of (D)-amphetamine sulphate on noradrenaline metabolism in discrete areas of rat brain

423P AMBROZI, L., BIRKMAYER, W., RIEDERER, P. & YOUDIM, M.B.H. L-Dopa and (–)-deprenil in the treatment of Parkinson's disease: a long-term study

424P RICHARDS, J.G. Autoradiographic evidence for the effects of specific uptake-inhibitors on the selective accumulation of [$^3$H]-5-HT by supraependymal nerve terminals and for the localization of binding sites for [$^3$H]-DLSD

425P FILLION, G., FILLION, M.P., JACOB, J. & ROUSSELLE, J.C. 5-HT and LSD high affinity binding sites to brain synaptosomal membranes

426P HILLIER, K., ROBERTS, P.J. & WOOLLARD, P.M. Catecholamine-stimulated prostaglandin synthesis in rat brain synaptosomes

427P HILEY, C.R. Ontogenesis of muscarinic receptor sites in rat brain

428P COLQUHOUN, D. Analysis of end-plate current fluctuations produced by acetylcholine and acetylmonoethylcholine in rat muscle

429P LOUBATIÈRES, A.L., RIBES, G. & VALETTE, G. Pharmacological agents and acute experimental hyperlactataemia in the dog

430P CHUBB, I.W. & RAINE, A.E.G. (introduced by Vaughan Williams, E.M.). Long-term effects of propranolol on tyrosine hydroxylase and dopamine-$\beta$-hydroxylase in the superior cervical ganglia of the rabbit

431P AUCLAIR, M.C., JOURDON, P., LECHAT, P. & LEMEIGNAN, M. Action of cytochrome C on transmembrane potentials of normal or hypoxic guinea-pig myocardial strips

431P FARRAR, D.G. & VERITY, ANNE. Analysis of the effects of isoxsuprine on guinea-pig atria and trachea

432P ALTMAN, J., Da PONTE, F. & WORCEL, M. Altered reactivity of stomach fundus smooth muscle in Okamoto spontaneous hypertension

433P CHEVILLOTTE, E., DEVYNCK, M.A., MEYER, P., PAPADIMITRIOU, A., ROUZAIRE-DUBOIS, B. & WORCEL, M. Molecular mechanism of post-nephrectomy uterine supersensitivity to angiotensin

434P LUGNIER, C., MICHON, T. & STOCLET, J.C. Evolution of aortic and cardiac cyclic AMP phosphodiesterase during the onset of mineralocorticoid hypertension in the rat

435P COHEN, Y., LINDENBAUM, A., PORQUET, D. & WEPIERRE, J. Evidence of central cardiovascular effects of intracerebroventricular isoprenaline in anaesthetized rat

435P NAHORSKI, S.R. & SMITH, BRENDA Characteristics and altered sensitivity of cerebr $\beta$-adrenoceptors assessed by [$^3$H]-propranolo binding

436P DUBOST, M., ESCOUSSE, A., MOUNIÉ, J. TRUCHOT, R. Peripheral effects of t amphetamine-type anorectic drugs: inhibition o catecholamine-induced lipolysis, respiration glucose utilization in the adipose tissue of man an rat

437P DUMEUR, G., HÜE, BERNADETTE, LWOFF J.M., MOURIES, M.A. & TREMBLAY, Activity of anorectic drugs (amphetamin amfepramone and UP 507–04) on two models o obesity in animals

437P MARCY, R. & QUERMONNE, M. Decremental skin conductance response in mic during iterative photostimulation; an attentio sustaining capacity model for psychopharm cological research

439P CLARKE, CORAL H., NICHOLSON, A.N. & STONE, BARBARA M. Effect of the 1, benzodiazepines, clobazam and triflubazam, on th sleep of man

439P SILVERMAN, A.P. Behavioural changes in rat suggesting drug-induced headache

440P FAGG, G.E., JORDAN, C.C. & WEBSTE R.A. The release of endogenous amino acids fro the cat spinal cord *in vivo*

441P LEWIS, D.A. & PARROTT, D.P. The effect o cortisol on $\alpha_1$-macroglobulin and $\alpha_2$-acute phase globulin in the arthritic rat

442P MILLER, P. & ROBSON, P. (introduced by James, G.W.L.). Potentiation of anaphylacti bronchoconstriction by non-steroidal anti-inflammatory agents

443P DELBARRE, B., SCHMITT, H. & SENON DANIELLE. Effects of activation of $H_1$ and H receptors on central cardiovascular structures i cats and on behaviour in chickens

444P DUCKWORTH, J.W., LANCE, J.W., LOR G.D.A. & MYLECHARANE, E.J. (introduced b Raper, C.). Histamine receptors in the crania circulation of the monkey

445P BURKA, J.F., EYRE, P. & HOLROYDE, M.C. Dopaminergic (co-mediator) modulation of relea of histamine and SRS-A in the calf

446P WHITTLE, B.J.R. Calcium and the inhibition histamine release from rat peritoneal mast cells non-steroid anti-inflammatory agents

447P VARGAFTIG, B.B. Effects of prostagland synthetase inhibitors and of other drugs o aggregation of plasma-free rabbit platelets carrageenan and by thrombin

448P ADOLPHE, M., DUNN, C.J., FONTAGNÉ, J., GIROUD, J.P., LECHAT, P., PAUBLANT, D. & WILLOUGHBY, D.A. Mitogenic activity of different types of non-immunological inflammatory exudates on macrophages in culture

448P CONOLLY, M.E., GREENACRE, J.K. & SCOFIELD, P. Dose-dependent desensitization of lymphocyte beta-receptor function after long-term culture with isoprenaline

450P ALRIC, R., LIGNON, F., LOUBATIÈRES, A.L., MANTEGHETTI, M. & PUECH, R. Non-saturable co-operative binding of tolbutamide to isolated islets of Langerhans

450P ISMAHAN, G., PARVEZ, H. PARVEZ, S. & RAZA-BUKHARI, A. Interrelations between MAO activity and carbohydrate metabolism

451P DEWS, P.B. Effects of drugs on suppressed responding

452P BOISSIER, J.R., DUMONT, C., OBERLANDER, C. & PETERFALVI, M. A comparison of circling behaviour induced in nitro-striatal lesioned rats after peripheral administration of indole derivatives

453P WADDINGTON, J.L. (introduced by Crow, T.J.). A behavioural model of the GABA-facilitating action of benzodiazepines: rotational behaviour after unilateral intranigral injection of chlordiazepoxide

454P KOOB, G., DEL FIACCO, MARINA & IVERSEN, SUSAN D. The behavioural effects of EOS-induced changes in substantia nigra GABA levels

454P BOISSIER, J.R., GODEFROY, F., SOUBRIE, P., THIEBOT, M.H. & WEIL-FUGAZZA, J. Platelet uptake of [$^{14}$C]-5-hydroxytryptamine in 'emotional' and 'non-emotional' rats

455P CURZON, G. & MARSDEN, C.A. Effects of $p$-chlorophenylalanine and $\alpha$-methyltryptophan on rat social behaviour

456P BOWERY, N.G. Reversal of the action of $\gamma$-aminobutyric acid (GABA) antagonists by barbiturates

458P BREUKER, E., DINGLEDINE, R. & IVERSEN, L.L. Evidence for naloxone and opiates as GABA antagonists

458P DAVIES, J. & DRAY, A. Actions of enkephalin and morphine on spinal cord and brain stem neurones

59P HILL, R.G. & PEPPER, C.M. The effects of morphine and metenkephalin on nociceptive neurones in the rat thalamus

60P BIRDSALL, N.J.M., BRADBURY, A.F., BURGEN, A.S.V., HULME, E.C., SMYTH, D.G. & SNELL, C.R. Interactions of peptides derived from the C-fragment of $\beta$-lipotropin with brain opiate receptors

462P ARMSTRONG, J.M. & LATTIMER, N. Prostaglandin $E_2$ released from the kidneys of genetic hypertensive rats contributes to the vasoconstrictor supersensitivity to angiotensin II *in vitro*

463P CARDINAL, D.C. & HILL, T.W.K. (introduced by Walker, J.M.G.). A device for controlling perfusion pressure

464P DAWES, P. & REDFERN, P.H. A simple and inexpensive method for the intracerebral administration of drug solutions to the conscious rat

464P CLARKE, G. & LINCOLN, D.W. Central and peripheral inhibition of the milk-ejection reflex: studies with $\mu$-adrenoceptor antagonists

465P FILE, SANDRA E., HYDE, J. & POOL, M. (introduced by Neal, M.J.). Effects of ethanol and chlordiazepoxide on social interaction in rats

466P de BONO, D.P., GORDON, J.L., MacINTYRE, D.E. & PEARSON, J.D. Active transport of adenine and adenosine by blood platelets and cultured endothelial cells

467P LEWIS, D.M. & WEBB, SANDRA N. Adrenaline on isotonic contractions of mammalian skeletal muscle

468P KRUK, Z.L., SMITH, L.A. & ZARRINDAST, M.R. Antagonism of responses to anorectics by selective receptor blockers

469P JENNER, P.G. & PYCOCK, C.J. Interaction of clonidine with dopamine-dependent behaviours in rodents

470P PILOT, MARIE-ANNE (introduced by D'Mello, A.). Indomethacin and acid secretion in the isolated perfused canine stomach

471P BOOT, J.R., DAWSON, W. & OSBORNE, D.J. The biological significance of prostaglandin-like substances released from immunologically challenged guinea-pig lungs

472P BOLAM, J.P., FORD-HUTCHINSON, A.W., SMITH, M.J.H. & WALKER, J.R. A sponge implantation test in the rat as a model for screening anti-inflammatory activity

472P BENDING, M.R., BENNETT, P.N. & ROWLAND, M. Pethidine pharmacokinetics in dog: dose and time studies

473P BARBHAIYA, R.H. & TURNER, P. Fluorimetric determination of ampicillin and cephalexin

474P BLAU, K., GOODWIN, B., WOODS, H.F. & YOUDIM, M.B.H. Phenylethylamine formation in perfused rat liver

475P CHICHMANIAN, R.M., KINSUN, I., MOULIN, M.A. & SAVINI, E.C. The effect of glucocorticoids on turn-over of acetylcholine and prostaglandins in the isolated guinea-pig ileum

## VOLUME 58 NUMBER 4 DECEMBER 1976

479 PERSSON, C.G.A. Inhibitory innervation of cat sphincter of Oddi

483 AZUMA, H., BANNO, K. & YOSHIMURA, T. Pharmacological properties of N-(3′,4′-dimethoxycinnamoyl) anthranilic acid (N-5′), a new anti-atopic agent

489 METCALF, G. & MYERS, R.D. A comparison between the hypothermia induced by intraventricular injections of thyrotropin releasing hormone, noradrenaline or calcium ions in unanaesthetized cats

497 GIACHETTI, A. & HOLLENBECK, R.A. Extravesicular binding of noradrenaline and guanethidine in the adrenergic neurones of the rat heart: a proposed site of action of adrenergic neurone blocking agents

505 DAY, M.D. & ROACH, A.G. Cardiovascular effects of dopamine after central administration into conscious cats

517 CHEN, C.S., GATES, G.R. & REYNOLDSON, J.A. Effect of morphine and naloxone on priming-induced audiogenic seizures in BALB/c mice

521 ROSS, S.B. Long-term effects of *N*-2-chloroethyl-*N*-ethyl-2-bromobenzylamine hydrochloride on noradrenergic neurones in the rat brain and heart

529 WONG, P.Y.D. & YEUNG, C.H. Inhibition by amiloride of sodium-dependent fluid reabsorption in the rat isolated caudal epididymis

533 CURZON, G. & FERNANDO, J.C.R. Effect of aminophylline on tryptophan and other aromatic amino acids in plasma, brain and other tissues and on brain 5-hydroxytryptamine metabolism

547 PARRATT, J.R. & STURGESS, R.M. The effect of a new anti-inflammatory drug, flurbiprofen, on the respiratory, haemodynamic and metabolic responses to *E. coli* endotoxin shock in the cat

553 STEINESS, EVA & VALENTIN, N. Myocardial uptake of digoxin in chronically digitalized dogs

561 BROWN, F. & REDFERN, P.H. Studies on the mechanism of action of amantadine

569 COTMAN, C.W., HAYCOCK, J.W. & LEVY, W.B. On the functional coupling of neurotransmitter uptake and release in brain

573 McDONALD-GIBSON, WENDY J., SAEED, S.A. & SCHNEIDER, C. The local antinociceptive and topical anti-inflammatory effects of propyl gallate in rodents

583 CONSTANTI, A. & NISTRI, A. Antagonism by some antihistamines of the amino acid-evoked responses recorded from the lobster muscle fibre and the frog spinal cord

593 DE JONG, W. & NIJKAMP, F.P. Centrally induced hypotension and bradycardia after administration of $\alpha$-methylnoradrenaline into the area of the nucleus tractus solitarii of the rat

599 HEDQVIST, P. Further evidence that prostaglandins inhibit the release of noradrenaline from adrenergic nerve terminals by restriction of availability of calcium

605 FAST, D. & TENENHOUSE, A. The effect of dibutyryl cyclic adenosine-3′,5′-monophosphate on protein secretion from the rat exocrine pancreas *in vitro*

613 BARLOW, R.B., BERRY, K.J., GLENTON, P.A.M., NIKOLAOU, N.M. & SOH, K.S. A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and in ileum at 29°C and 37°C

# INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac,* **50**, 3-23, may also be obtained. Specific points to note are:

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors **in alphabetical order**. A short title of not more than 50 letters should also be suggested.

2. **Statement** Papers are accepted only if accompanied by a statement that they have not been and will not be published in whole or in part in any other Journal.

3. **Summary** (2 copies) This should be a short summary of results and conclusions arranged in numbered paragraphs.

4. **Abstract** (2 copies) An abstract of about 100 words suitable for inclusion in an abstracting journal should be typed on a separate sheet, giving the author's name and address and the title of the paper.

5. **Figures** These should not be larger than A4 (206 x 294 mm) and may be in the form of original drawings, recorded tracings or high quality photographic prints made from them. Negative prints of kymograph tracings (black on white) should be provided, and both ordinates and abscissae should be calibrated. Lettering on figures should be inserted in pencil. Symbols which are to appear in figures or legends should be chosen from the following:

○ ● □ ■ △ ▲ ▽ ▼

Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction.

6. **Tables** These should not have more than 85 characters to a line (counting spaces between columns as 4 characters) and certainly not more than 110 characters to a line, unless absolutely unavoidable. **Figure legends** and **tables** should be typed on separate sheets of paper.

7. **References** These should be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the fourth edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should in addition, include the names of editors, the edition number, where appropriate, and the town of origin and name of publisher.

**Two page proofs** will be supplied, one of which may be retained by the authors. The other should be corrected immediately and returned to the Press Editor. Corrections should be kept to a minimum.

**Twenty-five reprints** will be supplied to the authors free of charge. Additional reprints can be purchased. A reprint order form, which should be returned promptly, will be sent out with the proofs. No reprints of abstracts are supplied.

**Short Communications** These may be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text and only one figure or table. The summary should be a single paragraph. An abstract and statement should be included.

# British Journal of Pharmacology

VOLUME 58 NUMBER 4 DECEMBER 1976

479 PERSSON, C.G.A. Inhibitory innervation of cat sphincter of Oddi

483 AZUMA, H., BANNO, K. & YOSHIMURA, T. Pharmacological properties of N-(3′,4′-dimethoxycinnamoyl) anthranilic acid (N-5′), a new anti-atopic agent

489 METCALF, G. & MYERS, R.D. A comparison between the hypothermia induced by intraventricular injections of thyrotropin releasing hormo[illegible] noradrenaline or calcium ions in unanaesthetized cats

497 GIACHETTI, A. & HOLLENBECK, R.A. Extravesicular binding of noradrenaline and guanethidine in the adrenergic neurones of the rat heart: a proposed site of action of adrenergic neurone blocking agents

505 DAY, M.D. & ROACH, A.G. Cardiovascular effects of dopamine after central administration into conscious cats

517 CHEN, C.S., GATES, G.R. & REYNOLDSON, J.A. Effect of morphine and naloxone on priming-induced audiogenic seizures in BALB/c mice

521 ROSS, S.B. Long-term effects of *N*-2-chloroethyl-*N*-ethyl-2-bromobenzylamine hydrochloride on noradrenergic neurones in the rat brain and heart

529 WONG, P.Y.D. & YEUNG, C.H. Inhibition by amiloride of sodium-dependent fluid reabsorption in the rat isolated caudal epididymis

533 CURZON, G. & FERNANDO, J.C.R. Effect of aminophylline on tryptophan and other aromatic amino acids in plasma, brain and other tissues and on brain 5-hydroxytryptamine metabolism

547 PARRATT, J.R. & STURGESS, R.M. The effect of a new anti-inflammatory drug, flurbiprofen, on the respiratory, haemodynamic and metabolic responses to *E. coli* endotoxin shock in the cat

553 STEINESS, EVA & VALENTIN, N. Myocardi uptake of digoxin in chronically digitalized dogs

561 BROWN, F. & REDFERN, P.H. Studies on t mechanism of action of amantadine

569 COTMAN, C.W., HAYCOCK, J.W. & LEVY W.B. On the functional coupling of neuro transmitter uptake and release in brain

573 McDONALD-GIBSON, WENDY J., SAEED S.A. & SCHNEIDER, C. The local antino ciceptive and topical anti-inflammatory effec of propyl gallate in rodents

583 CONSTANTI, A. & NISTRI, A. Antagonism b some antihistamines of the amino acid-evoke responses recorded from the lobster muscle fib and the frog spinal cord

593 DE JONG, W. & NIJKAMP, F.P. Central induced hypotension and bradycardia aft administration of α-methylnoradrenaline into the area of the nucleus tractus solitarii of the rat

599 HEDQVIST, P. Further evidence that prost glandins inhibit the release of noradrenaline fro adrenergic nerve terminals by restriction of ava ability of calcium

605 FAST, D. & TENENHOUSE, A. The effect dibutyryl cyclic adenosine-3′,5′-monophospha on protein secretion from the rat exocrine pancre *in vitro*

613 BARLOW, R.B., BERRY, K.J., GLENTO P.A.M., NIKOLAOU, N.M. & SOH, K.S. A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and in ileum at 29°C an 37°C

Printed in Great Britain by The Whitefriars Press Ltd., London and Tonbridge